MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE ROLE OF HYDROGEN SULPHIDE IN SEXUAL MEDICINE

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SUMMARY

Hydrogen sulphide (H_2S) is an endogenously produced gasotransmitter with a similar role as nitric oxide (NO) which has long been recognised as an important mediator in erectile physiology. Several studies have investigated the role of H_2S in erectile function and H_2S was found to exert definitive pro-erectile effects. The aim of this thesis is to elucidate the contribution of H_2S to erectile response and shed some light on the mechanism(s) involved, including any possible cross talk between H_2S and NO.

It was observed that NaHS, a H_2S-donor, significantly improved the magnitude of erectile response to cavernous nerve electrical stimulation in rats. This improvement was associated not only with an increase in the systemic H_2S concentration and H_2S biosynthesis in the corpus cavernosum (CC) of these rats but also with increased production of NO in both plasma and CC. The cross talk between H_2S and NO was evident in this tissue. Further *in vitro* studies revealed that H_2S increased endothelial nitric oxide synthase (eNOS) mRNA expression and cyclic guanosine monophosphate (cGMP) level. Moreover, H_2S also exerted an effect on the NO pathway downstream of NOS, namely increasing the expression of both the active and inactive forms of soluble guanylyl cyclase (sGC) β1 and stimulating the translocation of sGCα1 from the cytosol to the membrane. Overall, H_2S seems to play a 'supportive' role with respect to NO pathway in erectile physiology, amplifying NO signalling through dual action of increasing NO production and sensitizing the sGC towards NO. In addition, studies using castrated animals demonstrated that testosterone is not a requirement for the pro-erectile effect of H_2S; however, testosterone is clearly implicated in this cross talk. High testosterone level seems to favour the cross talk, with H_2S boosting NO production in this condition while low testosterone seems to cause H_2S to 'switch' to an NO-independent mechanism for its pro-erectile effect. Interestingly, H_2S seems to act as a backup when the NO pathway is compromised. Under condition of high NO (observed in animals treated with sildenafil), normal H_2S level and production were observed, while under

condition of low NO (observed in animals treated with NO synthase inhibitor L-NAME), high H_2S level was observed. Thus, shortage of NO can trigger the production of H_2S, which can in turn stimulate the production of NO. The finding from this study that exogenous H_2S seems to stimulate endogenous H_2S production also shed some light on the possible auto-regulation of H_2S through positive feedback.

The pro-erectile effect of H_2S was also likely to result from its attenuating effect on the RhoA/Rho-Kinase contractile pathway. In this system, H_2S was shown to downregulate the level of RhoA and Rho Kinase II (ROCK II) proteins which may have direct implication on corporal smooth muscle tone.

In summary, findings from this thesis work show that H_2S plays an important physiological role in erectile function. It is likely to exert its pro-erectile effects through multiple mechanisms of action including a complex cross talk with NO as well as modulation of the contractile, anti-erectile pathway.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AAT	aspartate (cysteine) aminotransferase
AAV	adeno-associated virus
AC	adenylyl cyclase
AOAA	aminooxyacetic acid
AS	argininosuccinate
ASL	argininosuccinate lyase
Asp	L-aspartate
ASS	argininosuccinate synthase
BH_4	tetrahydrobiopterin
cAMP	cyclic adenosine monophosphate
CBS	cystathionine β-synthase
CC	corpus cavernosum
CDO	cysteine deoxygenase
cGK	cGMP-dependent protein kinase
cGMP	cyclic guanosine monophosphate
CO	carbon monoxide
CSD	cysteine sulphinate decarboxylase
CSE	cystathionine γ-lyase
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EC	enzyme commison number
ED	erectile dysfunction
EDRF	endothelium-derived relaxing factor
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay

eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
H_2S	hydrogen sulphide
HRP	horseradish peroxidase
HSP	heat shock-related protein
hVSMCs	human vascular smooth muscle cells
IBMX	3-Isobutyl-1-methylxanthine
ICP	intracavernosal pressure
iNOS	inducible nitric oxide synthase
IP_3	inositol triphosphate
K_2HPO_4	potassium phosphate dibasic trihydrate
KH_2PO_4	potassium dihydrogen phosphate
$KHPO_4$	potassium hydrogen phosphate
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
LPS	lipopolysaccharide
MAP	mean arterial pressure
MBS	myosin binding subunit
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MPST	3-mercaptopyruvate sulphurtransferase
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate

NaHS	sodium hydrosulphide hydrate
NANC	non-adrenergic non-cholinergic
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_2	nitrogen dioxide
NO_3	nitrogen trioxide
NO_3^-	nitrate
NOS	nitric oxide synthase
NTC	no template control
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
ONOO	peroxynitrite
OP	open probability
PAG	DL-propargylglycine
PDE	phosphodiesterase
PE	phenylephrine
PGE1	prostaglandin E1
PKA	protein kinase A
PKB	protein kinase B
PKG	protein kinase G
RhoGDI	rho-guanine dissociation inhibitor
RhoGEFs	guanine nucleotide exchange factors
ROCK	rho kinase
RQ	relative quantitative value
rRNA	ribosomal ribonucleic acid
RSNO	s-nitrosothiols
SEM	standard error of mean
sGC	soluble guanylyl cyclase

SNP	sodium nitroprusside
SO	sulphite oxidase
SOD	superoxide dismutase
STZ	streptozotocin
$T_{1/2}$	half-life
TMP	total cellular membrane protein
TSMT	thiol S-methyltransferase
TST	thiosulphate:cyanide sulphurtransferase
VIP	vasoactive intestinal polypeptide
Zn	zinc

1. INTRODUCTION

1.1 Penile structure and innervation

The erectile tissue is comprised of two functional compartments namely the paired corpora cavernosa and corpus spongiosum. The corpora cavernosa consist of smooth muscle fibers intertwined in the extracellular matrix of collagen and elastin; they are surrounded by multiple interconnecting sinusoidal spaces called lacunae and eventually by a thick fibroelastic sheath, the tunica albuginea (Figure 1.1) (Lue, 2000). Arterial blood flow to the corpus cavernosum (CC) is provided by the cavernosal arteries through branches of multiple resistance helicine arteries which lead directly into the lacunae. Venous outflow from the corpus cavernosum is provided by subtunical venous plexus which drains blood from the lacunae into emissary veins that pierce through the tunica albuginea and eventually into the deep dorsal vein (Banya *et al.,* 1989; Porst and Sharlip, 2006). When the smooth muscles of the helicine arteries are relaxed, blood inflow to the lacunar spaces increases. Relaxation of the smooth muscle of the trabeculae then dilates the lacunae, allowing for the expansion of the erectile tissue against the tunica albuginea which in the process, compresses the subtunical venules against the tunica (the stretching of the tunica also compresses the emissary veins), restricting the venous outflow. Penile erection is achieved through this combined increase in arterial inflow and reduction in venous outflow; a process referred to as the veno-occlusive mechanism (Saenz de Tejada *et al.,* 1991). Full erection phase is achieved when the increase in intracavernous pressure (to around 100 mmHg from 10-15 mmHg in the flaccid state) lifted the penile body from its dependent position to an erect state. This is followed by the rigid erection phase where the pressure becomes suprasystolic (>120 mmHg) with the contraction of the perineal (ischiocavernosus) muscles (Dean and Lue, 2005).

The penis is innervated by both somatic (dorsal) and autonomic nerve fibers (Lue, 2000). In the pelvis, they merge to form cavernous nerves. The somatic nerves supply the penis with

sensory fibers and are therefore primarily responsible for penile sensation. They also supply the perineal skeletal muscles with motor fibers to facilitate the contraction of the pelvic floor smooth muscle which would help to increase the corporeal body pressure and subsequently help to achieve maximum rigidity and ejaculation (Kandeel *et al.,* 2001). The autonomic nerve supplies are comprised of parasympathetic and sympathetic branches, which are involved in the initiation and inhibition of erection respectively (Steers, 1994). The parasympathetic nerve fibers divide into two different nerve terminals upon entering the CC: 1) cholinergic (acetylcholine) nerve terminals at endothelial cells and 2) non-adrenergic, non-cholinergic (NANC) nerves ending at cavernosal smooth muscles (Adaikan *et al.,* 1991). Erection inducing/stimulatory neurotransmitters include those from central nervous system such as dopamine (via D2 receptors) (Andersson, 2001), melanocortins (via melanocortin receptors) (Martin *et al.,* 2002), serotonin (via 5-HT receptor 2C (Stancampiano *et al.,* 1994; Millan *et al.,* 1997)), glutamate (Zahran *et al.,* 2000), EP peptides (hexarelin peptide analogues), vasoactive intestinal polypeptide (VIP) (Ottesen *et al.,* 1984; Adaikan *et al.,* 1986); neurotransmitters from the peripheral nervous system such as acetylcholine (Andersson, 2001), and NANC such as nitric oxide (NO) (Burnett *et al.,* 1992; Burnett, 2002). The sympathetic nervous system mediates corporal vasoconstriction and smooth muscle contraction and therefore, has a role in maintaining penis in flaccid state as well as in mediating detumescence after orgasm. The sympathetic nerve fibers innervate cavernous smooth muscle (stimulating α1 adrenoceptors) and cavernous vessels (stimulating mostly α2 adrenoceptors in penile and cavernous arteries (Andersson and Wagner, 1995) and mostly β2 adrenoceptors in helicine arteries (Saenz de Tejada *et al.,* 1996)).

Generally, penile erection is associated with relaxation of the corporal smooth muscle and flaccidity with contraction. The relaxation of the smooth muscle in the penile vasculature is also as important in erectile physiology as cavernosal smooth muscle relaxation. A balance exists between smooth muscle contraction and relaxation and this is regulated for the most part through a complex interplay of autonomic neurotransmitters.

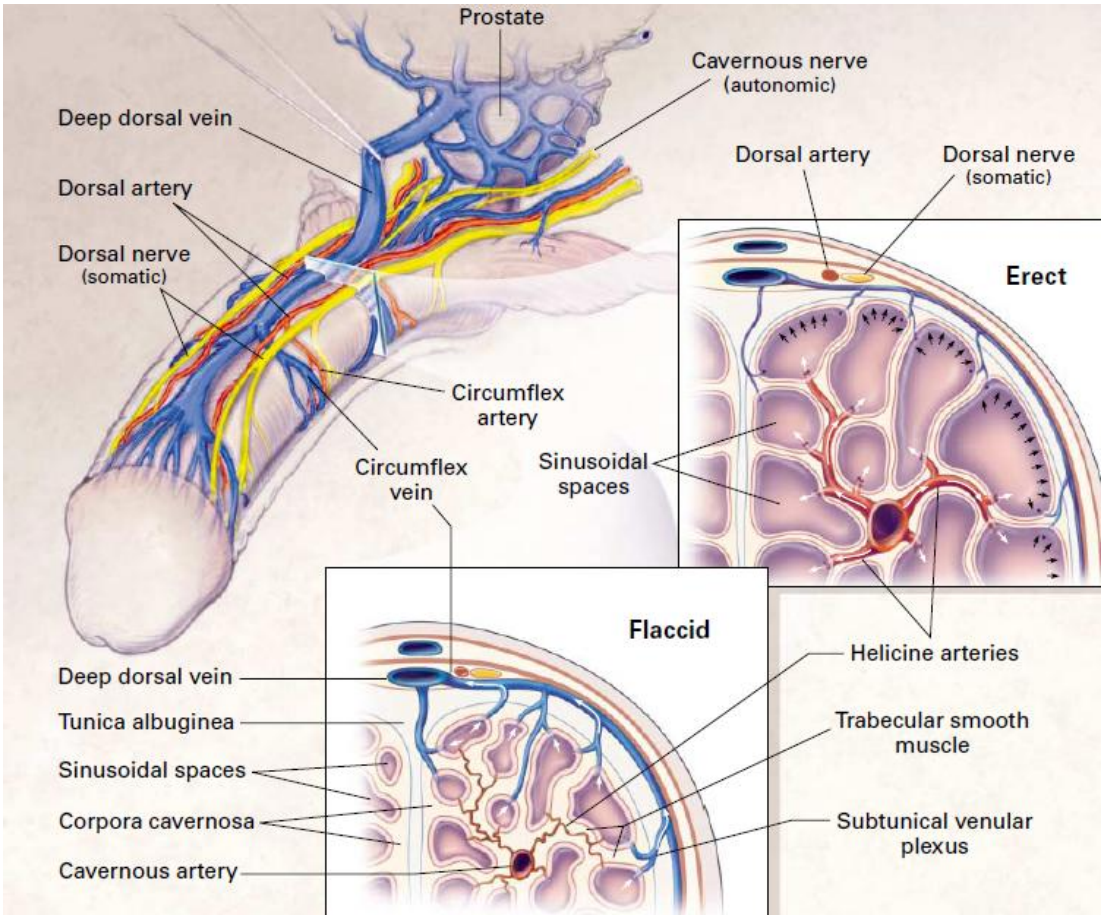


Figure 1.1 The anatomy and mechanism of penile erection. The cavernous (autonomic) nerves regulate penile blood flow during detumescence and erection while the dorsal (somatic) nerves are mainly responsible for penile sensation. The mechanisms of erection and flaccidity are shown in the inserts (Lue, 2000).

1.2 Erectile dysfunction

Erectile dysfunction (ED) is defined as the persistent inability to generate enough corporal body pressure necessary for vaginal penetration and/or the failure to maintain this level of rigidity in the penis until ejaculation for satisfactory sexual performance (Lizza and Rosen, 1999). It is a major health concern not only because it can significantly affect the quality of life but also because of its relatively high prevalence; the combined prevalence of ED (including mild moderate and complete) was estimated to be approximately 52% in men aged

between 40 to 70 years (Feldman *et al.*, 1994). It is also strongly associated with age and can be correlated with hypertension and heart disease (Feldman *et al.*, 1994). In fact, ED has been found to be a likely indicator of systemic vascular disease and may serve as an early warning for cardiovascular events such as myocardial infarct or stroke (Speel *et al*., 2003; Thompson *et al.*, 2005; Montorsi *et al.*, 2003b). The risk of ED was found to be 26/1000 every year and this incidence increases with age, hypertension, heart disease and diabetes (Johannes *et al.*, 2000). In the local context, ED is found to be common amongst Singaporean men; the prevalence for ED is 42% in forty-year old men and is as high as 77% in sixty-year old men (Tan *et al.*, 2003).

1.2.1 Pathophysiology of erectile dysfunction

Erectile physiology is an intricate interplay of vascular, neurologic and endocrine factors, making ED a multifactorial disorder that can be difficult to treat. The dysfunction can be psychogenic (performance anxiety related) or organic (e.g. as a result of hypertension, diabetes, hypercholesterolemia, etc). It can also be caused by pharmacological agents (such as anticholinergic, psychotropic, or antihypertensive medications) (Finger *et al.*, 1997; Crenshaw, 1996). Medications may get implicated in the development or exacerbation of ED in several ways: by inhibiting the central/peripheral nervous system, disturbing the hypothalamic-pituitary-gonadal axis, including androgen production and metabolism, altering the normal haemodynamics of hypogastric-cavernous arterial beds or by disturbing the control of the corporal vasomotor system (Goldstein and Krane, 1983). There has also been evidence that smoking is associated with vascular pathology (including atherosclerosis in the penile arteries) and may be a major risk factor for ED (Mannino *et al.*, 1994).

Organic cause of ED may be systemic such as endocrinal, vascular, neurological or local in nature. Systemic diseases such as diabetes mellitus (Feldman *et al.*, 1994; McCulloch *et al.*, 1980; Hidalgo-Tamola and Chitaley, 2009), renal failure (Palmer, 1999), cancer (Andersen,

1985; Cull, 1992) and chronic liver disease (Kew, 1988; Burra *et al.*, 2010) have been associated with ED. One of the most common forms of ED is related to vascular insufficiency, which includes arterial and venous insufficiency (Mulcahy, 2006). In arterial insufficiency, arterial supply is disrupted, usually from atherosclerosis or hypertension, resulting in poor penile perfusion. Venous insufficiency or leakage refers to inadequate trapping of blood in the corpora which may be caused by intrinsic abnormality in the smooth muscle, incomplete smooth muscle relaxation, or primary veno-occlusive dysfunction (Mulcahy, 2006). Chronic central nervous system disorders (e.g. Alzheimer's or Parkinson's disease, stroke), spinal cord injuries (trauma), or diabetes mellitus (Lue, 2000) may also affect the erectile pathway, reflexogenic erections and/or erectile response to psychogenic stimuli (Smith and Bodner, 1993; Courtois *et al.*, 1993). Similarly, local penile disorders such as Peyronie's disease (Hellstrom and Bivalacqua, 2000; Lopez and Jarow, 1993; Ralph *et al.*, 1996), phimosis (Alexander, 1993; Morgentaler, 1999), priapism (El-Bahnasawy *et al.*, 2002), or any congenital penile malformations/anomalies (Matter *et al.*, 1998) may interfere with normal erectile function resulting in ED.

1.2.2 Management of erectile dysfunction

There are several ways in which ED can be managed. These include psychological and behavioural counseling, drug therapy, the use of non-surgical devices (e.g. vacuum pump and constrictive ring), or surgery (e.g. repair of penile abnormality, penile prosthesis implantation, arterial revascularization or venous ligation) (Kandeel *et al.*, 2001). The choice of treatment should be considered based on the etiology behind the dysfunction. Generally, patients presenting with ED that is secondary to an underlying disease should be treated for the primary pathology, e.g. diabetic men with better glycemic control have been found to have lower odds ratio for ED (Fedele *et al.*, 1998). Some drug therapies that have been used with varying success, are reproductive hormones (androgen replacement in hypogonadal men presenting with ED) (Arver *et al.*, 1996; Mulhall, 2004), α2-adrenoceptor antagonist

(yohimbine) (Ernst and Pittler, 1998), centrally-acting drugs such as dopaminergic agonist (apomorphine) (Altwein and Keuler, 2001), and long-acting opiate antagonist (naltrexone) (Brennemann *et al.*, 1993). Besides systemic medications, local vasoactive agents can also be administered through direct intracavernosal injection (for example papaverine, phentolamine (Dinsmore, 1990), prostaglandin E1 (PGE1, alprostadil) (Virag and Adaikan, 1987), and VIP (Adaikan *et al.*, 1986)), or transurethral application (e.g. alprostadil) (Montorsi *et al.*, 2003a).

There are 11 known families of phosphodiesterase (PDE) enzyme systems, comprising of at least 60 distinct species, each differing in its kinetic properties, substrate specificity and tissue distribution (Bischoff, 2004). Phosphodiesterase type 5 (PDE-5) is the predominant cGMP metabolizing enzyme in penile arteries and CC, but it is also localised in lungs, platelet and vascular smooth muscle cells. Sildenafil, a classical PDE-5 inhibitor, approved in March 1998 and its successors have emerged as the first line of treatment and still are the most widely prescribed oral therapy for ED (Montorsi *et al.*, 2003a; Al-Shaiji and Brock, 2009), mainly because of their ease of use, efficacy and relatively low incidence of adverse effects (Fazio and Brock, 2004). Sildenafil is also known to be highly selective for PDE-5, compared to other PDEs (Bischoff, 2004). However despite its general efficacy, there remains a subpopulation of patients with ED (about 30-40%) who are resistant to this treatment regimen, necessitating a search for alternative approaches (Hatzimouratidis and Hatzichristou, 2005). Sildenafil works by inhibiting PDE-5, the enzyme that breaks down 3'5'-cyclic guanosine monophosphate (cGMP) - an important mediator in erectile physiology involved in smooth muscle relaxation - to 5'-GMP (Corbin and Francis, 1999), effectively increasing the cGMP level and thereby amplifying the cavernosal smooth muscle relaxation occurring after sexual arousal (Montorsi *et al*., 2003a). This means that the erectogenic effect of sildenafil relies very much on prior release of NO following sexual stimulation (the binding of NO to soluble guanylyl cyclase (sGC) increases the activity of the enzyme which would subsequently convert GTP to cGMP and increase the cGMP level (Ignarro, 2000)) and/or

possibly, the available cGMP pool in the body. Failure of sildenafil therapy that is observed in some patients may be attributed partly to insufficient production of NO (Rajfer *et al.,* 2002). Agents whose mechanism of action is independent of the NO/cGMP production may prove to be useful in pathological cases of ED where the NO/cGMP pathway is compromised.

1.3 Gasotransmitters

The neurotransmission in erectile physiology involves both sympathetic and parasympathetic pathways of the pelvic region. The sympathetic, anti-erectile neurotransmitter in human penile tissue is noradrenergic causing contraction of the CC muscle (Adaikan and Karim, 1981; Giuliano *et al.,* 1993); this transmitter is the main agent helping to keep the penis in rugose state. The parasympathetic neurotransmitter of erection to the cavernosum is not cholinergic (that is, not releasing acetylcholine, as it is in some other systems in the body) or adrenergic (that is not releasing noradrenaline). This type of neurotransmission was discovered and coined as NANC by Burnstock (Burnstock *et al*., 1964; Burnstock 1972) and subsequently was termed 'nitrergic' by Rand in 1992 (Rand 1992). The existence of NANC in rat anococcygeus and bovine retractor penis muscle was first reported by Gillespie (Gillespie 1972) and by Klinge and Sjostrand (Klinge and Sjostrand, 1974). Similarly, the identification of NANC neurotransmission in the human CC was first reported by Adaikan and Karim (Adaikan and Karim, 1978; Adaikan, 1979) and this neurotransmitter was confirmed to be nitrergic, releasing NO (Adaikan *et al.,* 1991).

Cellular signaling is usually initiated by the binding of factors or neurotransmitters to receptors on the plasma membrane. The resulting interaction between ligand and receptor generates intracellular second messengers which then relay the extracellular signals to different parts inside the cell, resulting in the modulation of cellular activities. The discovery of NO as an endothelium-derived relaxing factor (EDRF) in 1987 (Marsh and Marsh, 2000) represents the identification of cellular signaling mechanism that is receptor-independent. It

was observed that NO acted like a classical neurotransmitter, but with a different signaling mechanism. The term 'gasotransmitter' was then conceived to designate this molecule to distinguish it from classical neurotransmitters (Wang, 2002). Generally, to qualify as gasotransmitters, the molecules must possess the following characteristics: 1) they must be endogenously produced; 2) they must be freely permeable to membranes so that their effect(s) do not need to rely on membrane receptors; 3) their production and metabolism must be regulated; 4) at physiological concentration, they must have specific and well-defined function(s); and 5) regardless of whether their effects are mediated by intracellular second messenger or not, they should have specific molecular and cellular targets (Wang, 2002). Currently three gasotransmitters have been identified: nitric oxide, hydrogen sulphide (H_2S) and carbon monoxide (CO).

1.3.1 Hydrogen sulphide

1.3.1.1 Overview of H_2S

Decades of occupational health and environmental studies have described H_2S as a toxic pollutant that is detrimental to human health. This perspective has undergone a paradigm shift in recent years with the emergence of evidence for profound physiological effects of H_2S. Hydrogen sulphide seems to be able to exert a multitude of biological effects, having been implicated in inflammation (Zanardo *et al.,* 2006), antinociception (Distrutti *et al.,* 2006), myocardial ischaemia-reperfusion (Elrod *et al.,* 2007), cardiovascular pathology, shock/sepsis (Mok *et al.,* 2004; Collin *et al.,* 2005), pulmonary hypertension, and diabetes (Łowicka and Bełtowski, 2007). Essentially, H_2S is a lipophilic colorless gas with a 'rotten-egg' odor. It is also a weak acid; it can dissolve in water and dissociates to form HS^- and H^+ through the following reaction: $H_2S \leftrightarrow HS^- + H^+ \leftrightarrow S^{2-} + 2H^+$. The Henderson–Hasselbalch equation predicts that at the physiological pH of 7.4 and temperature of 37°C, 18.5% of the sulphide will exist as H_2S, with the remaining 81.5% as HS^- (Dombkowski *et al.,* 2004). It is still

currently unknown which of these molecules (H_2S, HS^- or S^{2-}) mediate the observed biological effects of H_2S (Whiteman and Moore, 2009).

1.3.1.2 Biosynthesis of H_2S

Most of the evidence for the physiological role of hydrogen sulphide is based on the observation that it is endogenously produced in tissues that are pertinent to its proposed roles (either as a vasorelaxant or neuromodulator). This means that the methodologies used to accurately measure this gas, which is both labile and present at relatively low concentration, must be rigorously assessed in order to avoid potential artifacts. Unfortunately, unlike NO which can be measured using its stable oxidation products (NO_2^- and NO_3^-), H_2S has no known stable or specific end product from its biosynthesis (SO_3^{2-} and SO_4^{2-} cannot be used to measure hydrogen sulphide production as they can also be formed from direct oxidation of L-cysteine with cysteine deoxygenase; refer to Figure 1.2). However, a majority of the studies (employing different analytical techniques) reported plasma H_2S in similar range (25-80 μM in rat and humans) with few exceptions (Whiteman and Moore, 2009), thereby suggesting that the measurements are likely to be credible.

Significant amount of H_2S is produced in most tissues in mammals including the penile tissue (Srilatha *et al.,* 2007), with higher production being observed in brain, liver, kidney, and the cardiovascular system (Doeller *et al.,* 2005; Zhao *et al.,* 2003). The majority of the endogenous H_2S is synthesised from L-cysteine by two pyridoxal-5'-phosphate (vitamin B6) dependent enzymes, cystathionine β-synthase (CBS, enzyme commission number (EC 4.2.1.22)) and cystathionine γ-lyase (CSE, EC 4.4.1.1) (Figure 1.2). The expression of these enzymes is tissue-specific; CBS is predominantly found in the central nervous system while CSE is expressed mainly in the liver, vascular and non vascular smooth muscles (Szabó, 2007). Human penile tissue homogenates express both CBS and CSE mRNA and protein (d'Emmanuele di Villa Bianca *et al.,* 2009). Another enzyme that can contribute to H_2S

biosynthesis is 3-mercaptopyruvate sulphurtransferase (MPST). Cysteine (aspartate) aminotransferase (AAT) first produces 3-mercaptopyruvate and L-glutamate by catalyzing the transamination between L-cysteine and α-ketoglutarate. The enzyme, MPST would then transfer sulphur from 3-mercaptopyruvate to sulphurous acid to generate pyruvate and thiosulphate which is then reduced to H_2S by another sulphurtransferase in the presence of reduced glutathione (Tanizawa, 2011). In this way, MPST (together with AAT) is found to significantly contribute to H_2S generation from L-cysteine in the presence of α-ketoglutarate in vascular endothelium of the thoracic aorta (Shibuya *et al.*, 2009a) as well as the brain (Shibuya *et al.*, 2009b). Hydrogen sulphide can also be synthesised from L-methionine through the trans-sulphuration pathway which involves the formation of homocysteine intermediate (Fiorucci *et al.*, 2006). Moreover, non-enzymatic reduction of elemental sulphur (inorganic source of H_2S) using reducing equivalents from glucose oxidation can also contribute to H_2S formation (Figure 1.3) (Szabó, 2007).

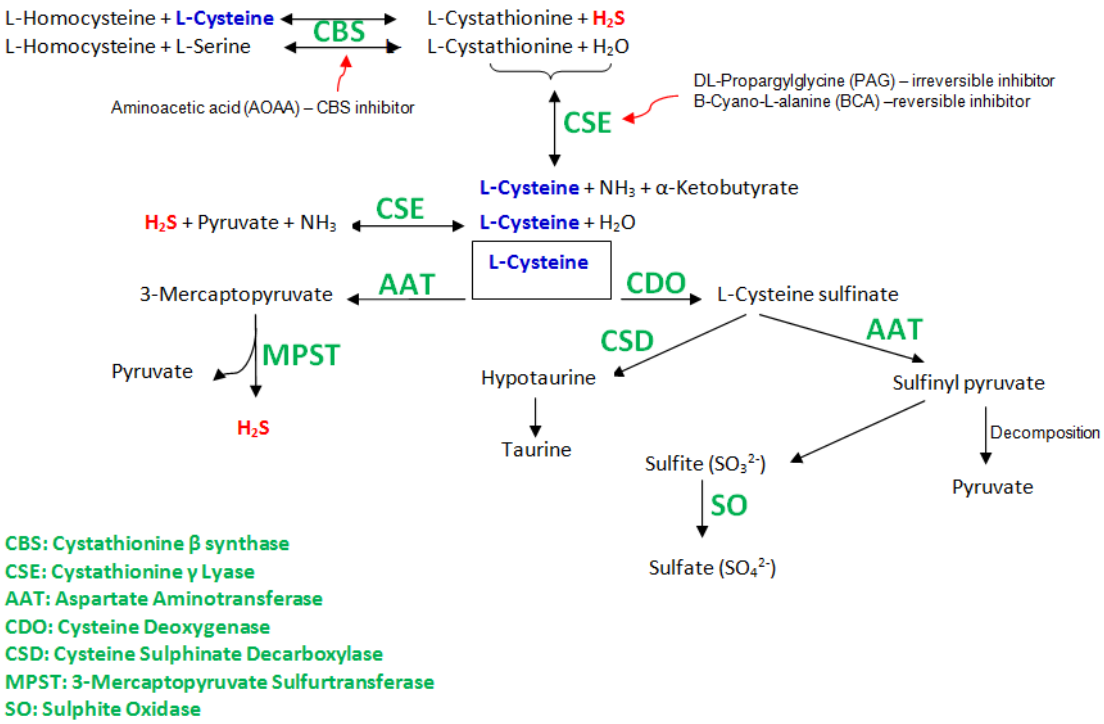


Figure 1.2 Enzymatic production of H_2S. (Compiled from (Wang, 2002; Szabó, 2007; Chen *et al.*, 2004; Kamoun, 2004; Li and Moore, 2008))

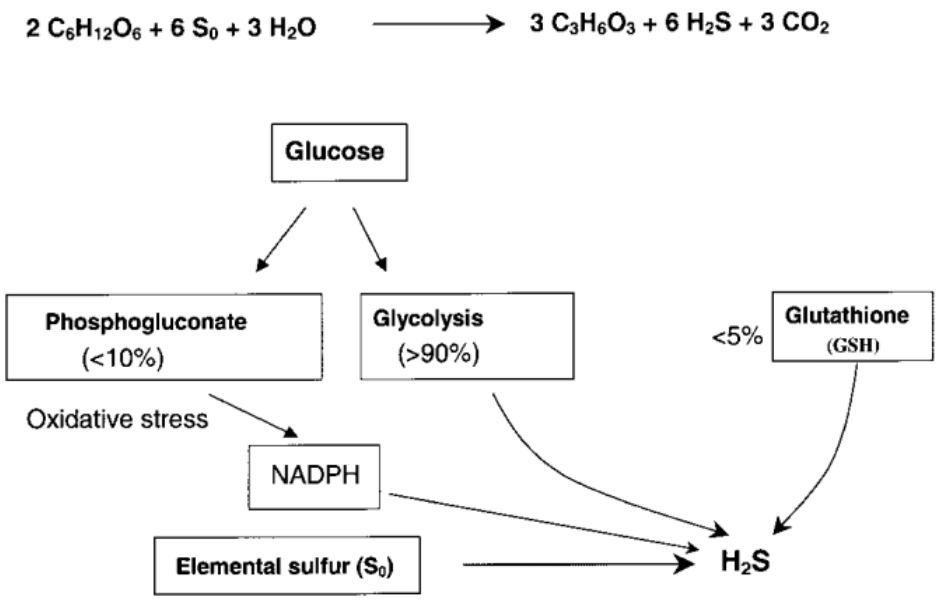


Figure 1.3 Non-enzymatic endogenous production of H_2S (Wang, 2002)

1.3.1.3 Metabolism of H_2S

Hydrogen sulphide is eliminated from the body mainly through the kidney either as conjugated or free sulphate. In the cell, catabolism of H_2S takes place in cytosol and mitochondria. It is metabolised in cytosol through a methylation process by thiol S-methyltransferase (TSMT) to methanethiol and dimethylsulphide (Furne *et al.*, 2001) and in mitochondria through an oxidation process to form thiosulphate, probably through either an enzymatic process catalyzed by superoxide dismutase (Searcy, 1996) or a non-enzymatic process as part of the mitochondrial respiratory electron transport (Łowicka and Bełtowski, 2007) (Figure 1.4). This thiosulphate would then be converted to sulphite by thiosulphate:cyanide sulphurtransferase (EC 2.8.1.1) and finally to sulphate by sulphite oxidase (SO). The H_2S can also be scavenged by metallo- or disulphide-containing molecules (e.g. oxidised glutathione) or by methemoglobin to form sulphhemoglobin (Wang, 2004). Hemoglobin is not only able to bind to H_2S; it can also bind to NO to form nitrosyl hemoglobin and to CO to form carboxyhemoglobin (Wang, 1998). In this way, it was

suggested that the bioavailability of one gas may be modulated by another as the binding by one would reduce the binding of the other gases to hemoglobin (Wang, 2002). This balanced metabolism at the cellular level means that H_2S produced endogenously under physiological condition is not toxic to the body as it gets rapidly oxidised in the mitochondria without accumulation (Wang, 2004). However, H_2S has a steep dose-response curve where the physiological effect transformed sharply into a toxic effect (Wang, 2002), as evidenced in rodent brain (Warenycia *et al.*, 1989) in which, the toxic level was less than double the endogenous level and H_2S intoxication also raised the endogenous level only by 57% (Mitchell *et al.*, 1993).

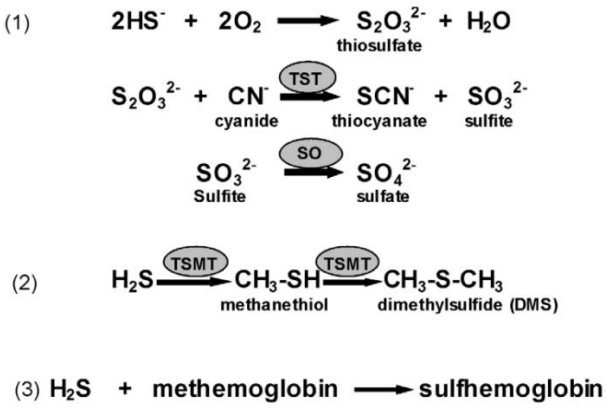


Figure 1.4 H_2S metabolism. (1) mitochondrial oxidation, (2) cytosolic methylation, (3) binding to methemoglobin. TST = thiosulphate:cyanide sulphurtransferase; SO = sulphite oxidase; TSMT = thiol S-methyltransferase (Łowicka and Bełtowski, 2007).

1.3.1.4 Roles of H_2S in erectile function

Preliminary study from our lab demonstrated that administration of sodium hydrosulphide hydrate ($NaHS.xH_2O$, a stable donor of H_2S) *in vivo* increased the penile length, penile perfusion and intracavernosal pressure (ICP) in non-human primates (Srilatha *et al.*, 2006). This is the first direct evidence for the pro-erectile effect of H_2S in CC. Since then, such facilitatory effects on erectile function have also been observed in other animal models; NaHS is shown to dose-dependently relax pre-contracted rabbit (Srilatha *et al.*, 2007) and human CC

(d'Emmanuele di Villa Bianca *et al.,* 2009) in organ bath studies while CSE inhibitor (DL-propargylglycine, PAG) is shown to lower the ICP in rats *in vivo* (Srilatha *et al.,* 2006). Similar to NaHS, L-cysteine - the H_2S precursor and CBS/CSE substrate - can also increase ICP and this effect is inhibited by PAG (d'Emmanuele di Villa Bianca *et al.,* 2009).

The classical inhibitor of adenylyl cyclase (AC), *cis*-N-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride, (MDL 12330A) is able to block H_2S-induced relaxation in pre-contracted rabbit CC but this inhibition appears to be incomplete (Srilatha *et al.,* 2007), suggesting thereby that while the cyclic adenosine monophosphate (cAMP) pathway is likely to be implicated in the mechanism of action of H_2S, it is not the only pathway and that there are other likely mechanism(s) that contribute(s) to the relaxant effect of H_2S. Furthermore, inhibition of endogenous H_2S production with PAG or the CBS inhibitor aminooxyacetic acid (AOAA) can also significantly increase the contraction induced by electrical field stimulation at different frequencies in rabbit (Srilatha *et al.,* 2007) and human (d'Emmanuele di Villa Bianca *et al.,* 2009) CC; this type of contraction is usually associated with detumescence. Taken together, the evidence suggests that the effects of H_2S may be twofold; being involved in 1) the relaxation of the corporal smooth muscle; and 2) the inhibition of the penile basal tone. The nature and site of H_2S effects (molecular/cellular/neurovascular) are unknown at this stage, but the finding has been significant considering that both impaired relaxation and increased contractility can contribute to ED.

In the human CC, CBS and CSE are found to be localised mostly in the vascular and trabecular smooth muscles (d'Emmanuele di Villa Bianca *et al.,* 2009). Moreover, the relaxant effect of H_2S appears to comprise of both endothelium-dependent and -independent components. This dual property of H_2S may have significant implication considering that one of the major contributing factors to penile vascular pathology in ED is endothelial dysfunction (Bivalacqua *et al.,* 2003). The novel H_2S pathway, by virtue of its lack of dependence on the integrity of the endothelium (which may be compromised in ED patients) for its production,

may aid in the relaxation of the cavernosum particularly in pathological conditions where endothelial nitric oxide synthase (eNOS) function is impaired (See Liaw *et al.*, 2011).

Studies on the vascular system show that H_2S causes vasoconstriction at low concentration but vasodilatation at high concentration (Kubo *et al* 2007a). This vasorelaxant effect involves potassium channel conductance, particularly K^+_{ATP} channel but not K_{Ca} or K_V (Zhao *et al.*, 2001) wherein H_2S can increase K^+_{ATP} channel currents, cause hyperpolarization (giving rise to the closure of voltage-dependent Ca^{2+} channel which decreased the intracellular Ca^{2+} to cause vasodilation (Brayden, 2002)) and significantly improve the K^+_{ATP} channel open probability (OP) (Tang *et al.*, 2005). The K^+_{ATP} channels are expressed in human CC (Insuk *et al.*, 2003); they have a functional role in penile resistance arteries (Ruiz Rubio *et al.*, 2004) and are important in the modulation of corporal smooth muscle tone and may well serve as targets for neurotransmitters (Christ, 2002). However, this K^+_{ATP}-dependent mechanism does not appear to be exclusive for H_2S since glibenclamide (K^+_{ATP} channel blocker) only partially inhibited H_2S-induced vasorelaxation (Zhao *et al.*, 2001). It is also proposed that the relaxant effect of H_2S may be mediated via a mechanism that involves metabolic inhibition, changes in intracellular pH and Cl^-/HCO_3^- channels (Kiss *et al.*, 2008).

The pro-erectile effect of H_2S seems to extend beyond its immediate relaxant activity in the penis. At the cellular level, H_2S is involved in modulating the level of anti-erectile proteins/factors which are pathophysiological in nature. In human vascular smooth muscle cells (hVSMCs) (Muzaffar *et al.*, 2008b) and pulmonary arterial endothelial cells (Muzaffar *et al.*, 2008a), H_2S can inhibit nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) activity and expression. This enzyme is a major source of superoxide (O_2^-) in the CC smooth muscle, being involved in the reduction of oxygen to superoxide (Babior, 2004). Elevated superoxide level is one of the known causative factors of ED (Jeremy *et al.*, 2006) and many factors associated with ED such as cytokines, angiotensin II and thromboxane A2 can also increase NADPH expression (Muzaffar *et al.*, 2005; Hotston *et al.*, 2007).

Superoxide anion can upregulate the expression of PDE-5 and react with NO to form the reactive oxygen species peroxynitrite (ONOO), which not only causes a reduction in the level of bioavailable NO but also causes tissue injury and alteration in the vascular tone (Figure 1.5) (Jones *et al.*, 2002). By reducing NADPH expression and activity, H_2S can help to abrogate the effects of superoxide anion especially under pathological conditions and boost the erectile capacity. The concentration at which H_2S exerts this inhibitory effect is much lower than that which causes relaxation (Shukla *et al.*, 2009), suggesting that the potential of H_2S lies not only in its acute pro-erectile effect but also in its longer term effect in suppressing the expression of proteins that may be up-regulated in ED.

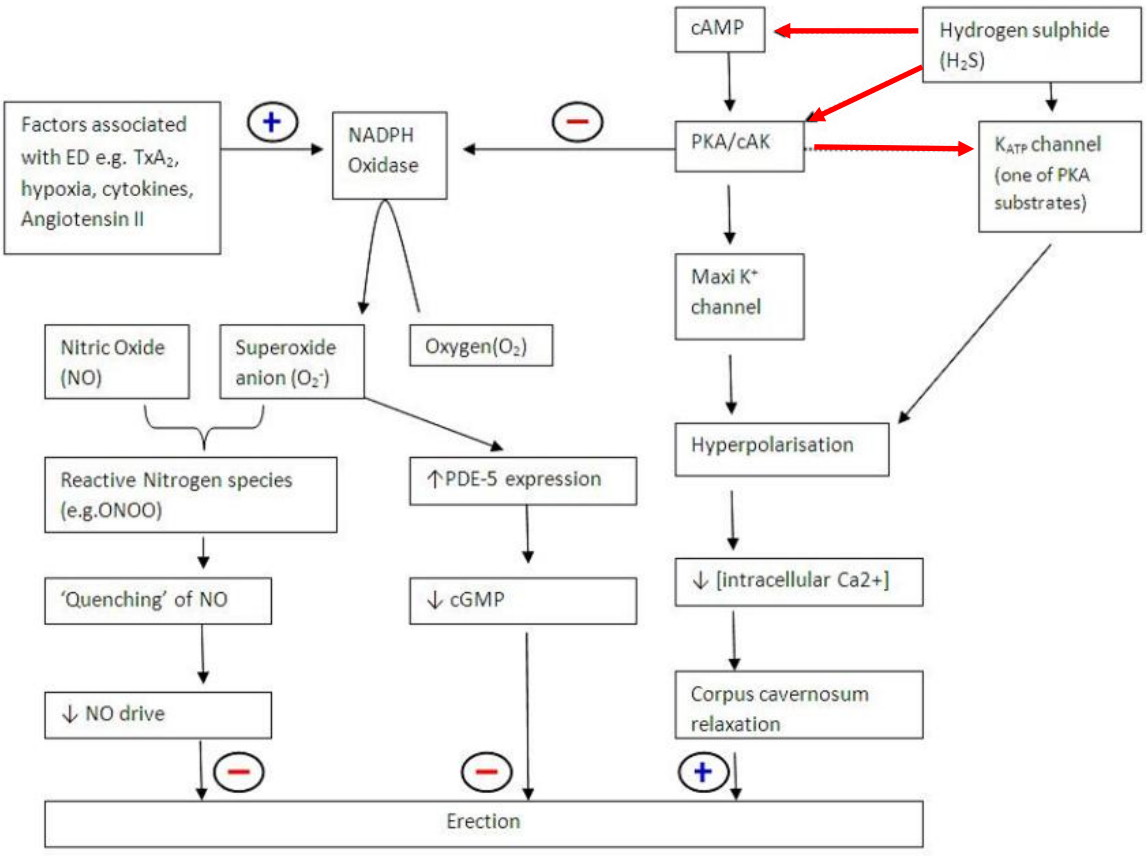


Figure 1.5 H_2S as an inhibitor of superoxide formation. (+) = stimulation; (–) = inhibition. Red arrows represent possible hypothetical pathways that have not been proven (Hotston *et al.*, 2007; Muzaffar *et al.*, 2008b; Jeremy *et al.*, 1999; Shukla *et al.*, 2009)

1.3.2 Nitric oxide

1.3.2.1 Overview of NO

With the arrival of the industrial revolution, natural gases such as NO were viewed by the public as atmospheric pollutant and toxic waste. Nitric oxide is emanated from industrial processes and motor car exhausts (Bruckdorfer, 2005); it can also be generated by lightning in the troposphere (Levine *et al.,* 1984) or released by nitrifying bacteria in the soil (Conrad, 1996). Nitric oxide is essentially an odourless, colourless, lipophilic and soluble diatomic gas which is also a free radical (Bruckdorfer, 2005). In 1980, Furchgott and Zawadski discovered an agent of endothelial origin, that relaxed the arterial smooth muscle and not knowing the identity of the agent at that time, they referred to it as the EDRF (Furchgott and Zawadzki, 1980). It was only seven years later that the identity of EDRF was revealed to be NO (Ignarro *et al.,* 1987b; Ignarro *et al.,* 1987a). Nitric oxide is now recognised as an important ubiquitous intercellular signalling molecule in many tissues. It has antiplatelet aggregatory and anti-inflammatory properties, both pro- and anti-angiogenic activity and can decrease leukocyte adhesion (Wang, 2004).

1.3.2.2 Biosynthesis of NO

Nitric oxide is biosynthesised from L-arginine (an amino acid that is present at high concentration in the blood, extracellular fluid and inside the cell) through a series of redox reaction involving several co-factors with specific binding sites, and producing L-citrulline as by-product (Bruckdorfer, 2005). The co-factors include tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and calmodulin (Figure 1.6) (Li *et al.,* 2009b). The guanidine nitrogen of L-arginine is oxidised, eventually forming the nitrogen of NO while the oxygen in NO is derived from molecular oxygen (Li *et al.,* 2009b). This reaction is catalyzed

by nitric oxide synthase (NOS), of which there are three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and eNOS (Wang, 2004). These isoforms are found on different chromosomes with different subcellular localization and mode of regulation. The two isoforms, nNOS and eNOS, are constitutively expressed in a cell-specific manner, producing low amounts of NO (in pico to nanomolar range) (Moncada *et al.,* 1991) and can be activated by calcium binding to calmodulin (Li *et al.,* 2009b). They are regulated mainly at the post-translational stage (Bivalacqua *et al.,* 2002). Under certain pathological condition, eNOS activity may be altered, for example eNOS is inhibited in diabetic hyperglycemia through a post translational modification involving protein kinase B (PKB, also known as Akt) (Du *et al.,* 2001). Shear stress is also thought to activate eNOS, possibly through the activation of calcium channels (Lin *et al.,* 2000). Inducible NOS is a calcium-independent isoform of NOS; its expression can be induced by inflammatory mediators or immunological stimuli such as cytokines or bacterial lipopolysaccharide (LPS), producing higher amounts of NO (in nano to micromolar range) (Wang, 2004).

It is known that nNOS generates NO in the nerves of the central and peripheral autonomic nervous system. Nitric oxide is released from the NANC nerves that innervate the visceral smooth muscle (Adaikan et al., 1991; Rand and Li, 1995). The NO released mediates smooth muscle relaxation and is involved in regulating bronchodilation, sphincter function, and gastrointestinal motility (Wang, 2004). The nNOS is located mainly in the mitochondria and cytoplasm of the cell (Jobgen *et al.,* 2006) while eNOS is expressed by endothelial cells; it is located within the caveolae of the plasma membrane but is also present in the cytoplasm (Jobgen *et al.,* 2006). On the other hand, iNOS is produced by macrophages, neutrophils and vascular smooth muscle cells (Bishop-Bailey *et al.,* 1997) and is mostly localised in the cytoplasm (Jobgen *et al.,* 2006). Almost all cell types are able to recycle citrulline back into arginine through the argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) pathway (Wu and Brosnan, 1992). This recycling helps to ensure that there is sufficient concentration of arginine for production of NO. The main site of NO production in human CC

is in the terminal branches of the cavernous nerves that supply the erectile tissue (Burnett *et al.*, 1993) where NO is formed through the activity of nNOS in the NANC neurons (Burnett *et al.*, 1992; Cartledge *et al.*, 2001) and eNOS in the endothelium (Hurt *et al.*, 2002).

Biosynthesis of NO is dependent on the availability of the substrate L-arginine and the various co-factors (in particular BH_4 (Ignarro, 2000)) that are needed for the NOS enzyme activity. Even though the concentration of L-arginine within and outside the cell is usually well above the saturation point of the enzyme, under conditions where endothelial function is impaired, L-arginine level may be a limiting factor (Bruckdorfer, 2005). Biosynthesis of NO may also be partly influenced by the presence of naturally occurring NOS inhibitors inside the cell or in the blood e.g. asymmetric dimethylarginine or L-monomethyl arginine which is a naturally occuring competitor of L-arginine (Li *et al.*, 2009b). Nitric oxide can also be produced through a non-enzymatic process. Nitrite, on its own has negligible relaxant activity but under acidic condition, it can be reduced back to NO. The conjugated acid of nitrite can react with another nitrite to generate N_2O_3, which then releases NO (Zweier *et al.*, 1999).

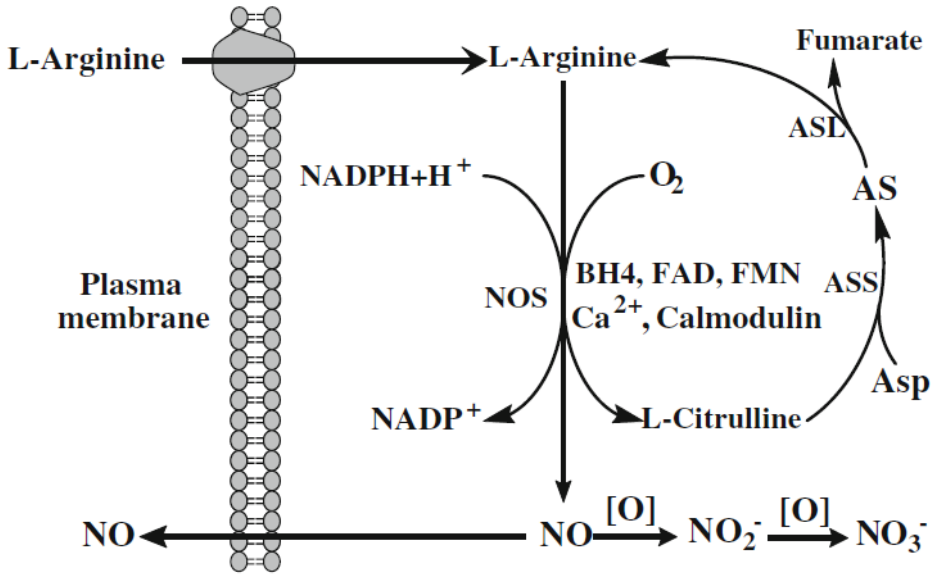


Figure 1.6 Synthesis of NO from L-arginine. AS = argininosuccinate; BH4 = tetrahydrobiopterin; ASL = argininosuccinate lyase; ASS = argininosuccinate synthase; Asp = L-aspartate (Li *et al.*, 2009b).

1.3.2.3 Metabolism of NO

The mode and rate of NO metabolism in the body depends on several factors, including the concentration of NO itself, its diffusibility and the surrounding concentration of other bioreactants. Having a neutral charge, NO has high diffusion capacity, being able to diffuse in aqueous solution, across membranes and over long distances in tissues (Kelm, 1999). When exposed to oxygen, NO can produce reactive nitrogen oxide species e.g. nitrogen dioxide (NO_2) and nitrogen trioxide (NO_3). Nitric oxide also undergoes auto-oxidation to release NO_2 in aqueous solution which can undergo further reaction to form nitrite (NO_2^-) and nitrate (NO_3^-) (Wang, 2004).

The auto-oxidation kinetics of NO in aqueous solution is dependent on its concentration (Ford *et al.*, 1993) and therefore the half-life ($T_{1/2}$) of NO is not a constant value and is in fact inversely related to NO concentration (Kelm, 1999). This means that the $T_{1/2}$ of NO becomes longer as NO gets more dilute. As NO moves away from its site of origin, it will get diffused and its concentration will drop with distance. With a lower NO concentration, its lifetime increases and this results in a higher effective 'bioavailability' of NO, allowing it to react with other biological molecules e.g. plasma proteins, oxyhemoglobin, or sGC enzyme (Wink and Mitchell, 1998). Nitrite and nitrate are considered stable end products of NO metabolism and they are both excreted by the kidneys. Collectively, their level can be used as a measure of NO synthesis in the body (Kelm, 1999).

Endogenous biotransformation of NO occurs through different metabolic routes. Essentially, it can react rapidly with superoxide anion to form peroxynitrite (Huie and Padmaja, 1993). In the blood, NO can also get oxidised by oxyhemoglobin to produce nitrate and methemoglobin (Kelm, 1999). The cysteine residue of globin in hemoglobin functions as a reversible carrier of NO for delivery to tissues (Allen *et al.*, 2009). Reacting with thiols, NO may form S-nitrosothiols (RSNO) with a longer $T_{1/2}$ than NO, which is important for its stability and

transport; RSNO also serves as a stable reservoir of NO (Wang, 2004). Superoxide dismutase (SOD) – scavenger of superoxide anion – can also protect NO since NO is inactivated by superoxide and in this way, SOD can indirectly enhance the availability and duration of action of NO (Kelm, 1999; Wang, 2004).

1.3.2.4 Roles of NO in erectile function

As mentioned earlier, NO is an important neurotransmitter of human penile erection (Adaikan *et al.,* 1991). Nitric oxide released from nerve endings and endothelial cells activates sGC which mediates increased conversion of guanosine triphosphate (GTP) to cGMP (Ghalayini, 2004). Cyclic GMP governs many aspects of cellular function through its interaction with cGMP-dependent protein kinases, cyclic nucleotide phosphodiesterases or cyclic nucleotide gated-ion channels (See Ignarro, 2000). It can also stimulate protein kinase G (PKG), which would in turn initiate the phosphorylation of membrane-bound proteins at K^+ channels (See Francis *et al.,* 2010). This leads to K^+ ions outflow into the extracellular space, hyperpolarizing the cells (Figure 1.7) to bring about closure of L-type Ca^{2+} channels with a resultant drop in intracellular Ca^{2+} ions concentrations (Lue, 2000).

Physiologically, intracellular Ca^{2+} and calmodulin activate the myosin light chain kinase (MLCK), whose function is to catalyse the phosphorylation of myosin light chain (MLC) and induce actin-myosin interaction, which is necessary for cavernous smooth muscle contraction in the non-erect state (Gao *et al.,* 2001). The decrease in intracellular Ca^{2+} brought about by NO leads to reduced activation of MLCK, resulting in decreased phosphorylation of the MLC and reduced actin-myosin interaction, eventually leading to corpus cavernosal relaxation and erection. Vasoconstrictors like endothelin-1 (ET-1) and norepinephrine stimulate the activity of phospholipase C to increase inositol triphosphate (IP_3) and diacylglycerol (DAG), resulting in increased intracellular Ca^{2+} phosphorylation of MLC and smooth muscle contraction - NO

reverses this process by increasing cGMP level (Figure 1.7) (See Porst and Sharlip, 2006; Saenz de Tejada, 2000; Mills *et al.*, 2001).

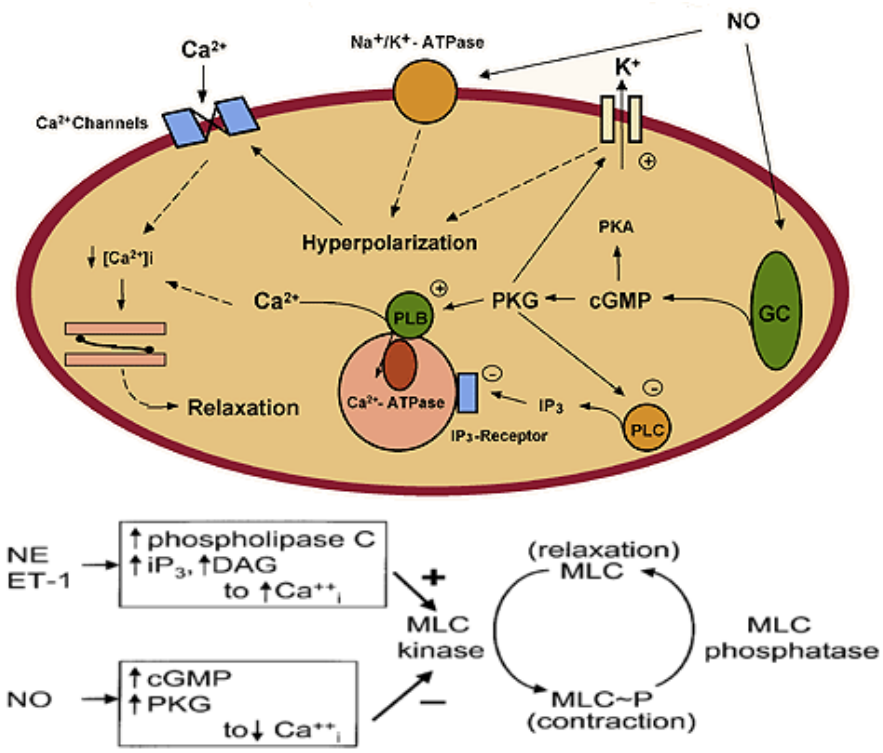


Figure 1.7 Relaxation of penile smooth muscle via the NO/cGMP pathway (Porst and Sharlip, 2006; Saenz de Tejada, 2000; Mills *et al.*, 2001).

1.3.2.5 RhoA/Rho-kinase in contractile mechanism

The degree of actin-myosin interaction that is essential for smooth muscle contraction depends on the phosphorylation state of MLC. This, in turn depends on two enzymes: 1) the Ca^{2+}-calmodulin activated MLC kinase which phosphorylates MLC (as discussed in the previous section); and 2) the Ca^{2+}-independent MLC phosphatase (MLCP) which dephosphorylates MLC (Sauzeau *et al.*, 2000). In other words, smooth muscle contraction can be mediated in two ways: by increasing the intracellular cytosolic Ca^{2+} and its subsequent activation of MLCK or by increasing the Ca^{2+} sensitivity of the contractile apparatus by inhibiting the activity of MLCP. Conversely, relaxation of smooth muscle can result from a

decrease in cytosolic Ca^{2+} concentration and/or 'Ca^{2+}-desensitization' of the contractile apparatus (Somlyo and Somlyo, 1994; Somlyo, 1997).

At the cellular level, RhoA/Rho-kinase signalling pathway acts on the MLCP to mediate contraction at a constant Ca^{2+} concentration. RhoA is a small monomeric GTPase. In resting smooth muscle, most of the RhoA is in the cytosol where it is bound to guanosine diphosphate (GDP) and is rendered inactive. When the GDP is converted to GTP, RhoA is activated and translocated into the plasma membrane (Gong *et al.,* 1997a). Activated RhoA can stimulate Rho-kinase (a serine/threonine kinase), which would then phosphorylate the myosin binding subunit (MBS) of MLCP. Phosphorylated MLCP is the inactive form of MLCP and therefore, it will promote higher levels of phosphorylated MLC, actin-myosin interaction and smooth muscle contraction. On the other hand, inhibition of Rho-kinase helps to increase MLC phosphatase activity, MLC dephosphorylation and smooth muscle relaxation (Mills *et al.,* 2001).

In the dynamic equilibrium of erectile response, there is evidence that the RhoA-dependent Ca^{2+} sensitization/contraction can be inhibited by NO/cGMP/PKG signalling. At the cellular level, cGMP through cGMP-dependent protein kinase (cGK) phosphorylates and inhibits the activity of RhoA. As further confirmations, sodium nitroprusside (SNP) – an NO donor - is found to inhibit the translocation of RhoA to the plasma membrane; a process which is required for its activation (Sauzeau *et al.,* 2000) and detumescence. Similarly, the NO-induced increase in ICP is also shown to be potentiated by prior treatment with Rho-kinase inhibitor (Mills *et al.,* 2002).

1.3.3 Cross talk between H_2S and NO

Several groups have attempted to elucidate the relationship between H_2S and NO but while the evidence generally points to the existence of a cross talk between the two

gasotransmitters, the exact nature of the interaction is difficult to characterise accurately. On one hand, there is evidence that the two gases are synergistic in their actions/effects (Hosoki *et al.*, 1997). On the other hand, they can regulate each other's production (Zhao *et al.*, 2001). Some groups reported that H_2S can regulate NO production, for example: studies by Kubo and co-workers showed that NaHS inhibited all three isoforms of NOS; however this inhibition was reversed with increasing concentration of NOS co-factor, BH_4 (Kubo *et al.*, 2007b). The same group also observed that while NaHS inhibited the activity of recombinant eNOS, this inhibition was limited to the vasoconstrictor activity of H_2S (which occurs at low concentration of H_2S) because overall, H_2S still causes a dose-dependent relaxation of pre-contracted aortic tissue (Kubo *et al.*, 2007a). Furthermore, H_2S can also modulate NOS substrate availability by down-regulating the transporter for L-arginine (Geng *et al.*, 2007). Interestingly, there are also reports that NO can affect endogenous H_2S biosynthesis. Exogenous NO has been shown to increase CSE activity, possibly through direct interaction with CSE protein which contains 12 cysteines, the potential substrate for nitrosylation (Zhao *et al*., 2001). Additionally, NO may also modulate CSE substrates' availability, considering that NO has been shown to stimulate the uptake of cystine (a known CSE substrate) (Li *et al.*, 1999).

It therefore, appears that the relationship between the H_2S-NO cross talk and its functional end result (contraction/relaxation) is complex and context-dependent, possibly because H_2S may have multiple mechanisms of action. For example, the concentration dependent contractile/relaxant activity of H_2S may be mediated through different mechanisms or modulation of the same mechanism. This means that the difference in the microenvironment conferred by different tissue/organ system is likely to be an important factor and results pertaining to H_2S effects on one organ system may not be readily extrapolated to other organ system. Unfortunately, most of the investigations that explore the cross talk between H_2S and NO were done in the vascular system; there is currently very limited information of this cross talk on non-vascular smooth muscle cells, particularly on the penile tissue (which is

comprised of both vascular and non-vascular smooth muscles). The cross talk between H_2S and NO in erectile physiology is of particular interest considering the importance of the NO pathway in this system.

2. RESEARCH INTEREST AND OBJECTIVES

Research on gasotransmitters is an interesting field because it defies the long standing notion that communication between cells has to be limited by intracellular distances and receptor-ligand interaction. Being gasotransmitters, H_2S and NO seem to share a lot of similarities in their action. Previous studies done in this lab also show that H_2S, like NO, possesses pro-erectile properties but its mechanism(s) of action is still largely unknown (Srilatha *et al*., 2006).

As such, the objectives of this study are to:

1. Verify that H_2S is endogenously produced in the animal model
2. Investigate the effects of H_2S on erectile function *in vivo* using rat models
3. Investigate the mechanism(s) of facilitatory action of H_2S, including:
    - Involvement of the second messenger system cAMP and cGMP
    - Interaction with RhoA/Rho-kinase pathway
    - Hormonal influence
4. Investigate the interaction between H_2S and NO at different points in the pathway, that is:
    - Production of NO
    - Expression of NOS
    - Expression of sGC

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs

Sodium hydrosulphide hydrate (NaHS; H_2S donor; Cat # 161527), Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; non-specific nitric oxide synthase inhibitor; Cat # N5751) and DL-propargylglycine (PAG; CSE inhibitor; Cat # P7888) were purchased from Sigma Aldrich (St Louis, MO, USA). Testosterone cypionate and sildenafil citrate were purchased from Pfizer (Kent, UK). For *in vivo* treatment, all drugs except testosterone were dissolved in water. For *in vitro* studies using tissue/primary cell culture, NaHS and sildenafil were dissolved in Dulbecco's modified eagle's medium (DMEM).

3.1.2 Chemicals

All primers were synthesised by Sigma Aldrich. The kit for cGMP and cAMP enzyme immunoassay (EIA) was purchased from Cayman chemicals (Cat # 581021 and 581001 respectively). The medium used was DMEM1152 from Sigma Aldrich which contains L-glutamine, 4500 mg/L glucose, 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and was supplemented with 3.7 g/L of sodium bicarbonate (US Biological Cat # S4000). The chemiluminescent immunoassay kit for testosterone measurement was purchased from Siemens.

Other chemicals/items/kits used are as follows (in alphabetical order):

Items	Purchased from	Catalogue number
0.5% Trypsin-EDTA	Gibco (Invitrogen)	#15400
0.5 M Tris-HCl buffer pH 6.8	Biorad	#161-0799
1.5 M Tris-HCl buffer pH 8.8	Biorad	#161-0798
2-Propanol (Isopropyl alcohol)	Sigma Aldrich	#I9516
30% Acrylamide/Bis solution, 29:1 (3.3%C)	Biorad	#161-0156
3-Isobutyl-1-methylxanthine (IBMX)	Sigma Aldrich	#I7018
Agarose	Biorad	#161-3102
Ammonium persulfate (APS)	Biorad	#161-0700
Antibiotic:antimycotic solution	Gemini Bio-Products	#400-101
Baneocin	Sandoz GmbH	N.A.
Baytril	Bayer	N.A.
Blocking grade blocker non fat dry milk	Biorad	#170-6404
Blue/Orange 6x loading dye	Promega	#G190A
Bovine serum albumin (BSA)	Sigma Aldrich	#A-3311
Bradford reagent	Sigma Aldrich	#B6916
Carprofen (Rimadyl)	Pfizer	N.A.
Cell lytic MT solution	Sigma Aldrich	#C3228
Chloroform	Sigma Aldrich	#25668
DNA ladder 100bp	Promega	#G210A
DNA ladder 50bp	Promega	#G4521
DNaseI	Ambion	#AM2222
DNaseI buffer	Ambion	#AM8170G
Ethanol	Fisher Scientific	#E/0650DF/17
Ethylenediaminetetraacetic acid (EDTA) disodium salt	1st Base	#BIO-1050
Flavin adenine dinucleotide disodium salt hydrate (FAD)	Sigma Aldrich	#F6625
Foetal bovine serum (FBS)	Gibco (Invitrogen)	#10270-106
Gel red	Biotium	#41003
Glycine	Biorad	#161-0718
Heparin	Hanlim Pharm	N.A.
Immun-Blot PVDF membrane	Biorad	#162-0177
Iron (III) chloride ($FeCl_3$)	Sigma Aldrich	#F7134
Laemmli sample buffer	Biorad	#161-0737
L-cysteine	Sigma Aldrich	# 168149
Membrane protein extraction kit	Promokine	# PK-CA577-K268-50
Methanol	Sigma Aldrich	#34860
Microamp optical 98-well reaction plate	Applied Biosystem	#4306737
Mini trans blot filter paper	Biorad	#1703932
N-(1-Naphthyl)ethylenediamine dihydrochloride (NED)	Sigma Aldrich	#222488
N,N-dimethyl p-phenylenediamine dihydrochloride (NNDPD)	Sigma Aldrich	#D4139
Nitrate reductase (NAD[P]H)	Sigma Aldrich	#N7265-10UN
Phosphate buffered saline (PBS)	1st Base	#BUF-2040-10x1L
Phosphoric acid (H_3PO_4)	Sigma	#P5811
Potassium dihydrogen phosphate (KH_2PO_4)	Merck	#4531473410
Potassium phosphate dibasic trihydrate (K_2HPO_4)	Sigma Aldrich	#P9666

Precision plus protein standards	Biorad	#161-0374
Prostaglandin E1 (PGE1)	Sigma Aldrich	#P7527
Protease inhibitor cocktail	Sigma Aldrich	#P8340
Pyridoxal 5′-phosphate hydrate (PLP)	Sigma Aldrich	#P9255
RNase docontamination solution (RNaseZap®)	Ambion	#AM9780
Sildenafil citrate (Viagra)	Pfizer	N.A.
Sodium Azide (NaN_3)	Fluka Biochemika	#25668
Sodium dodecyl sulphate (SDS)	Biorad	#161-0301
Sodium nitrite ($NaNO_2$)	Sigma Aldrich	#S2252
Stripping buffer	Thermoscientific	#21059
Sulphanilamide	Sigma Aldrich	#S9251
Superscript III First strand synthesis system for RT-PCR	Invitrogen	#18080-051
SYBR Green PCR master mix	Applied Biosystem	#4309155
Tetramethylethylenediamine (TEMED)	Biorad	#161-0801
Transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol)	Self-reconstituted	N.A.
Trichloroacetic acid (TCA)	Sigma Aldrich	#T9159
Tris	Biorad	#161-0715
Tris/Borate/EDTA (TBE) buffer (89 mM Tris pH 7.6; 89 mM boric acid; 2 mM EDTA)	Vivantis	#PC0724-10x
Tris/Glycine/SDS buffer (25 mM Tris, pH 8.3; 192 mM Glycine, 0.1% SDS)	Biorad	#161-073
TritonX-100	Biorad	#161-0407
Trizol	Invitrogen	#15596-026
Trypan blue solution	Sigma Aldrich	#T8154
Tween20	Chemical Reagent	#T2008687
Zinc acetate	Sigma Aldrich	#383317
β-Mercaptoethanol	Biorad	#161-0710
β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH)	Sigma Aldrich	#N1630

Table 1. List of reagents, chemicals and kits used

3.2 Experimental Methods

Young adult (8-9 weeks old) male Sprague-Dawley (SD) rats were obtained from Laboratory Animal Centre, National University of Singapore (NUS) and given access to standard rat chow and water *ad libitum*. All experimental procedures involving laboratory animals were undertaken in accordance with established International Guiding Principles for Animal Research. The followed protocols were also duly approved by Institutional Animal Care and Use Committee (IACUC) of NUS.

3.2.1 Cell culture

3.2.1.1 Media preparation

The DMEM1152 (as described in section 3.1.2) was reconstituted with 10% FBS and 10 mL/L of antibiotic-antimycotic solution (containing penicillin G sodium salt, streptomycin sulphate and amphotericin B) and sterile-filtered by passing through 0.22 µm filter membrane. The prepared medium was then stored at 4°C until use.

3.2.1.2 Isolation of rat erectile tissue

For tissue collection, rat was first euthanised with an overdose of sodium pentobarbital euthanasia solution (200 mg/kg) which was administered intraperitonially. The penis was excised, and the connective tissue and fat were removed. The tissue was collected in the reconstituted DMEM medium and kept in ice. Subsequent steps after tissue collection were carried out in the sterile environment of a class II biosafety cabinet, fitted with high efficiency particulate air (HEPA) filter. The rat CC culture was prepared using the method by Krall *et al.* (Krall *et al.,* 1988) with some modifications (Carosa *et al.,* 2010; Zhao and Christ, 1995; Christ *et al.,* 1992).

3.2.1.3 Primary culture of rat corpus cavernosum smooth muscle

The CC was carefully separated from the fascia, glans penis and tunica albuginea, washed thrice in ice-cold sterile PBS, and then transferred to the DMEM medium. It was then cut into small strips (approximately 1 mm^3 in dimension). The explants were cultured in a 75 cm^3 tissue culture flask for 4-6 days at 37°C in a fully humidified CO_2 incubator (5% CO_2 and 95% O_2). When the explants had grown and became attached to the substratum as observed under a microscope, additional DMEM was added. Following this stage, the cells would

migrate from the explants and undergo proliferation. The medium inside the flask was replaced with fresh solution every 4 days. Upon reaching > 90% confluency, the cells were either used for experiment or sub-cultured. They were detached by treatment with 0.05% trypsin and 0.02% EDTA for 5-10 minutes at 37°C. Once complete detachment was observed, equal volume of the medium was added such that the activity of trypsin ceased. The suspension was centrifuged at 500 g for 5 minutes, washed in sterile PBS twice and resuspended in the reconstituted medium. Manual cell count, to determine the total number of cells was carried out with a hemocytometer (triplicate readings were made from each cell suspension sample). Trypan blue exclusion test was also carried out simultaneously to determine cell viability. The split ratio used for the subculture was adjusted according to the cell number such that each 175 cm^2 flask contained approximately 0.7-0.8 x 10^6 cells, an optimal seeding density required for cell growth.

Only cells with viability of > 90% were used for experiments. In order to preserve corporal tissue biology in its physiological state, only cells in the early passages (passage 1-3) were used. It was observed that cells from older passages (passage 5 onwards) showed abnormal morphology and an obvious decline in viability (< 70%). As confirmed by earlier studies elsewhere, cells at passage 1-3 also retain their normal cellular properties (including spindle-shaped morphology (Christ *et al.,* 1992; Campos de Carvalho *et al.,* 1993), calcium and potassium channel function (Christ *et al.,* 1993; Fan *et al.,* 1995; Zhao and Christ, 1995), PGE1- and forskolin-induced cAMP formation (Palmer *et al.,* 1994)).

Characterization of rat CC in primary cultures is usually done through immunostaining of α-actin, which is a specific marker for smooth muscle (Guidone *et al.,* 2002). This has been performed in this lab previously and it was established that the followed protocol can successfully generate relatively pure rat CC primary culture (Srilatha, 2003).

3.2.1.4 Trypan blue exclusion assay

This test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess cell membranes that are intact and therefore, are able to exclude the trypan blue dye in contrast to dead cells (without intact cell membranes), which take up the dye. In this test, a viable cell will appear with a clear cytoplasm when observed under a microscope, whereas a non-viable cell will have a blue cytoplasm. After the adherent cells were trypsinised and centrifuged, they were resuspended in serum-free DMEM. Equal parts of the cell suspension and 0.4% trypan blue solution were mixed. The mixture was allowed to incubate for 3 minutes at room temperature and then loaded into a hemocytometer for manual counting under a microscope. Stained (non-viable) and unstained (viable) cells were counted separately.

Percentage of viable cells = (Number of viable cells/ total number of cells) x 100%

3.2.2 Experimental protocol to investigate the involvement of second messenger cGMP and cAMP in H_2S action

Rat CC primary cultures at passage 1-3 were used for this component. A total of 0.25 x 10^6 cells were plated onto each well on a 6-well-plate in reconstituted DMEM and incubated overnight at 37°C. The cells were allowed to grow (attached to the substratum) and reach confluence. The medium was removed the following day. The cells were washed in PBS and conditioned in plain DMEM for 30 minutes before 250 µM of IBMX (non-specific PDE inhibitor) was added to each well to prevent the breakdown of cyclic nucleotides. The cells were then treated as detailed in Table 2 below.

	Treatment	Dose	Incubation condition
1	control (vehicle)	N.A.	30 minutes; 37°C; 5% CO_2; 95% O_2
2	NaHS	0.01-10 mM	
3	sildenafil citrate	1-100 µM	

Table 2. *In vitro* treatment of rat CC primary culture for cGMP and cAMP measurement

3.2.2.1 Measurement of cGMP and cAMP concentration

At the end of the incubation period, either cGMP or cAMP concentration was measured using competitive enzyme immunoassay in accordance with the manufacturer's (Cayman chemical) instruction. This assay is based on the competition between the free cXMP (where cXMP represents either cGMP or cAMP) present in the sample/standard with cXMP conjugated with acetylcholinesterase (AChE, cXMP tracer) for a limited amount of cXMP-specific rabbit antibody (cGMP antiserum) binding sites. The concentration of free cXMP varies while that of the tracer is held constant and therefore, the amount of cXMP tracer that binds to the antibody would be inversely proportional to the amount of free cXMP present in the samples.

The medium was first removed and the cells were lysed with 0.1 M HCl for 20 minutes at room temperature. Upon inspection under microscope that most of the cells had been lysed, the cells were scraped. The suspension was mixed with a pipette until it was homogenous and then centrifuged at 1000 g for 10 minutes. The supernatant was decanted into a clean test tube. The samples were then acetylated (using potassium hydroxide (KOH) and acetic anhydride) to enhance the sensitivity of the assay before they were transferred into the enzyme-linked immunosorbent assay (ELISA) plates and incubated with the cXMP tracer and antiserum for 18 hours at 4°C. The complex formed between the rabbit antibody (cXMP antiserum) and cXMP would bind to the anti-rabbit IgG that had been pre-coated on the ELISA plates (Figure 3.1). At the end of the incubation period, the plates were emptied and washed 5 times with the wash buffer. Ellman's reagent was reconstituted and added to each well. The mixture was incubated for 60-90 minutes in the dark, in an orbital shaker (IKA® KS260basic, Germany). The Ellman's reagent contained substrates to the enzyme cholinesterase. The reaction between AChE and its substrates generated products with a distinct yellow colour and strong absorption (optical density) at 412 nm. The colour intensity of the product formed was measured using a spectrophotometer (Bio-Tek μQuant, USA) at 410 nm to determine the concentration of cXMP in the sample. Protein concentration in each

sample was quantified using Nanodrop (ND-1000, Nanodrop Technologies). The cGMP and/or cAMP concentration in each sample was expressed as pmol per mg of protein. Each experiment was carried out in triplicate. The samples were also diluted appropriately (10-20 x for cAMP measurement and 2-6 x for cGMP measurement) to ensure that the readings fell within the range of the standard curve.

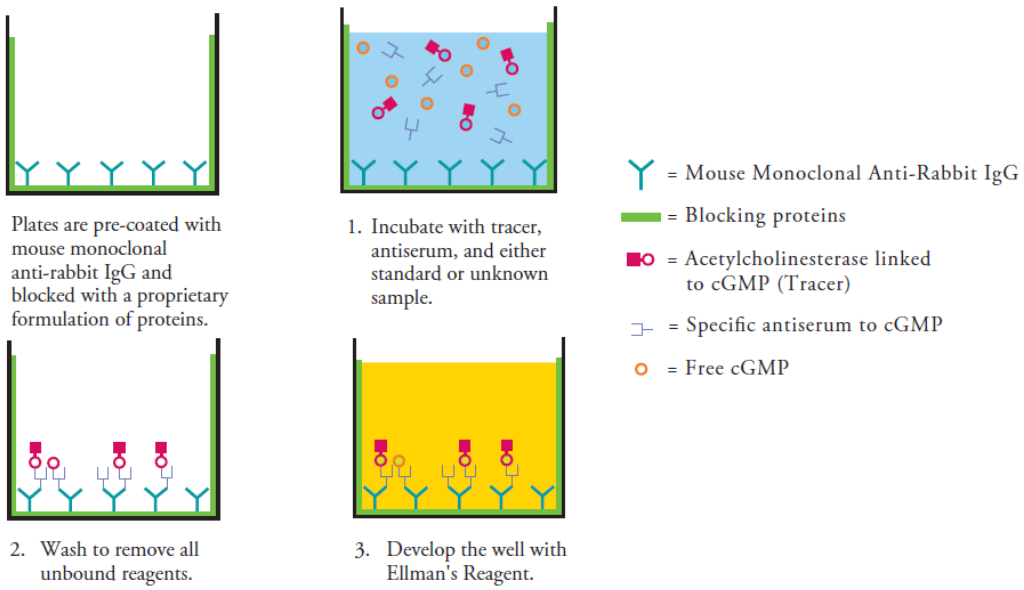


Figure 3.1 Schematic diagram of the colorimetric competitive EIA for cGMP measurement. The absorbance of the final product is directly proportional to the concentration of the bound cGMP tracer and inversely proportional to the concentration of free cGMP. A similar process takes place for cAMP measurement.

3.2.3 Experimental protocol to investigate effects of H_2S on erectile function *in vivo*

Age-matched rats were randomly assigned to 5 groups (n=7 per group), namely: 1) non-treated control, 2) sildenafil (0.7 mg/kg, oral, daily), 3) NaHS (0.8 mg/kg, ip, daily), 4) L-NAME (30 mg/kg, oral, daily), 5) PAG group (50 mg/kg, ip, twice-weekly). All treatments were administered for 10 weeks (Figure 3.2). The doses for different treatment regimens used were selected based on the existing literature, that employed the same drug (Balbinott *et al.*, 2005; Zhu *et al.*, 2007). At the end of 10 weeks, measurement of intracavernosal pressure and

mean arterial pressure (MAP) was done (please refer to section 3.2.3.1 below). The animals were then euthanised and the required samples (CC and blood) were collected. The blood was collected in a tube containing heparinised saline (250 U/ml of heparin) and centrifuged at 1500 g at 4°C for 15 minutes (Argmann and Auwerx, 2006). Upon collection, these samples (including plasma separated from the blood) were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Body weight of rats was also measured before and at the end of the treatment period.

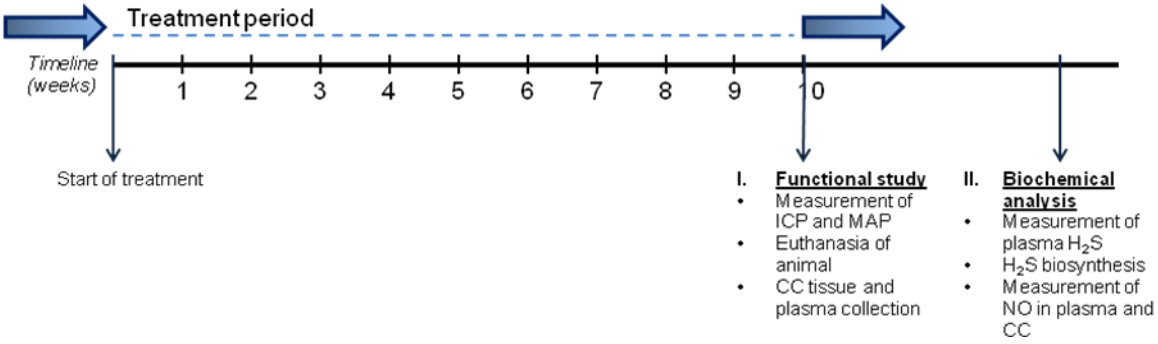


Figure 3.2 Schematic representation of experimental protocol for *in vivo* study

3.2.3.1 Measurement of Intracavernosal pressure

The procedure used for electrical stimulation and measurement of erectile response was a modification of the method described by Quinlan (Quinlan *et al.*, 1989) and Dail (Dail *et al.*, 1989). The rat was anesthetised with intraperitoneal injection of ketamine (75 mg/kg) and xylazine (10 mg/ml) mix and placed in supine position on a heating pad set at 37°C to maintain the body temperature, as thermoregulation may be compromised in rats under anesthesia (Wixson *et al.*, 1987). A longitudinal incision of about 3 cm was made in the neck region. The left external jugular vein was cannulated with PE10 tubing, connected to a syringe for infusion of drug (supplemental anesthesia where necessary as well as sodium pentobarbital for euthanasia at the end of the procedure). Tracheotomy was performed next to

assist respiration (Di Loreto, 2009). This is particularly important to prevent hypoxia, as this condition has been known to affect the H_2S pathway. The trachea was identified around the midline of the neck right underneath the muscular tissue. A small orifice was made in the trachea in between two cartilaginous rings and the catheter was inserted such that the bevelled end headed towards the lungs. The cathether and the trachea were tied together so as to secure the cathether in position. The right common carotid artery was cannulated with PE50 tubing, which was connected to a pressure transducer (AD Instruments) for measurement of the (systemic) MAP. A midline incision was made in the lower abdominal region to pack the testicles into the abdomen and dissect the lateroprostatic space to expose the pelvic plexus, pelvic nerve and cavernous nerve (Figure 3.3a) (Martinez-Pineiro *et al* 1994). The penile crus was exposed through a transverse perineal incision and separation of the overlying ischiocavernous muscle. A 27 G needle was connected through PE10 tubing to the pressure transducer. This needle was then inserted into the corpus cavernosum for monitoring/measurement of the intracavernosal pressure (Figure 3.3b). Bipolar platinum electrodes attached to a Grass Instruments stimulator (Warwick, USA) were hooked to the cavernous nerve. The lateral lobe of the prostate was manipulated to create free space underneath the cavernous nerve and ensure that its contact with the electrode was isolated. The following stimulatory parameters were used: 2 V at frequency of 20 Hz for 30-45 s (Srilatha and Adaikan, 2006), as they were found to give the most optimal response. All the needles and syringes used in this procedure were filled with heparinised saline to prevent blood clot formation and prior to each experiment, the pressure transducers were calibrated with a mercury manometer. The magnitude of erectile response was expressed as ICP normalised to MAP (considering that different drugs affect blood pressure differently, ICP/MAP ratio constitutes a more objective parameter to measure erectile response than ICP alone; this ratio has been used by other groups and is well recognised as a surrogate marker of erectile function (Park *et al.,* 2009)).

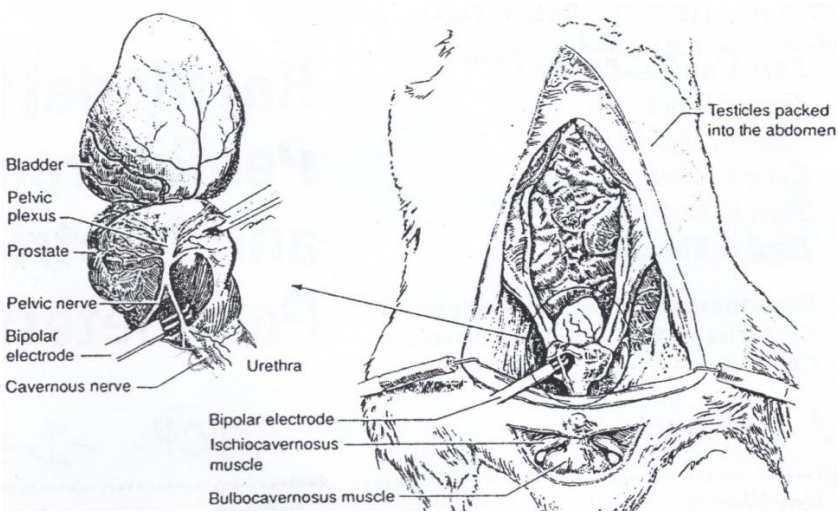


Figure 3.3a Animal preparation and the pelvic plexus (Martinez-Pineiro *et al.*, 1994)

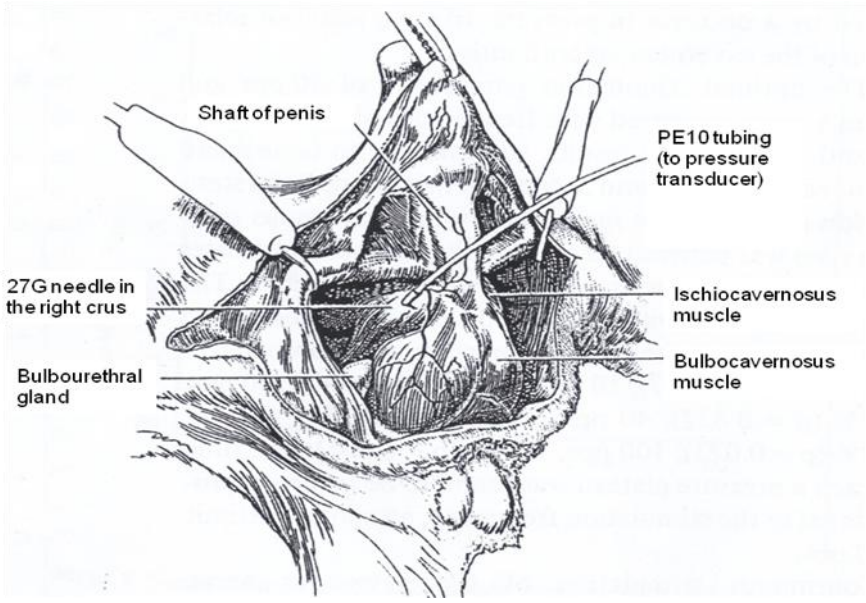


Figure 3.3b Perineal anatomy of the rat (Martinez-Pineiro *et al.*, 1994)

3.2.4 Experimental protocol to investigate effects of H_2S on biochemical parameters *in vivo*

3.2.4.1 Measurement of H_2S production (CBS/CSE activity) in corpus cavernosum

The H_2S-synthesizing activity in the corpus cavernosum was measured by the modified method of Stipanuk and Beck (Stipanuk and Beck, 1982). In this assay, the tissue was supplied with the substrate and co-factors for the enzyme CBS/CSE. Any H_2S released by the tissue was quantified and the rate of production of H_2S was taken as a measure of CBS/CSE activity in the tissue. The test sample, 0.1 g of the corpus cavernosum tissue was thawed and homogenised (Ultra-turrax, IKA® T10 basic) in 2 ml of 100 mM ice cold phosphate buffer ($KHPO_4$; pH 7.4). This buffer was prepared by mixing 100 mM K_2HPO_4 solution with 100 mM KH_2PO_4 solution in the ratio of 10:3 to achieve the correct pH. The tissue homogenate was centrifuged at 19000 g for 30 min at 4°C and the supernatant obtained was assayed. To 430 µl of the homogenate, 30 µl saline, 20 µl of 0.05 M PLP (final concentration in 500 µl reaction mixture: 2 mM) and 20 µl of 0.25 M L-cysteine (final concentration: 10 mM) were added sequentially in a vial (that was tightly sealed with a double layer of parafilm) and placed in ice until the reaction was initiated by transferring the vial containing the reaction mixture from ice to a water bath set at 37°C. The vial was left in the water bath for 30 minutes. Zinc acetate (1%, 250 µl) was then added to trap the evolved H_2S followed by treatment with TCA (10%, 250 µl) to stop the reaction. Subsequently, 133 µl of 20 mM NNDPD in 7.2 M hydrochloric acid (HCl) and 133 µl of 30 mM $FeCl_3$ in 1.2 M HCl were added sequentially and the absorbance of the resulting solution was measured spectrophotometrically at 670 nm, using a 96-well microplate reader (Bio-Tek µQuant, USA). As a control, 430 µl phosphate buffer was used in place of the tissue homogenate. All samples were assayed in duplicate. The H_2S concentration in the sample was calculated against a standard calibration curve of NaHS (3.125–250 µM) and the results were expressed as µmol of H_2S formed per gram of protein per hour. The protein concentration in each sample was

determined using nanodrop (ND-1000, Nanodrop Technologies). The calibration curve was prepared on the same day of sample processing.

3.2.4.2 Measurement of plasma H_2S concentration

The H_2S concentration in the plasma was assayed as described by Mok and co-workers (Mok *et al.*, 2004). The samples were centrifuged at 3000 rpm for 5 minutes after thawing and 100 µl of the plasma was added to a tube filled with 0.25 ml of 1% zinc acetate followed by addition of 400 µl of distilled water. The H_2S present in the sample will be chemiadsorbed by zinc acetate and transformed into stable zinc sulphide, which can then be recovered by extraction with water. To this, 133 µl of NNDPD in 7.2 M HCl and 133 µl of $FeCl_3$ in 1.2 M HCl were added sequentially and the final reaction mixture was allowed to incubate for 10 minutes at room temperature. In presence of $FeCl_3$ in a strongly acidic condition, the extracted sulphide reacts with NNDPD to yield methylene blue. At the end of the incubation period, 0.25 ml of 10% TCA was added to deproteinate the samples. The reaction mixture was then centrifuged at 19000 g for 5 minutes at 4°C and decanted into a 96-well plate where its absorbance was measured at 670 nm using a microplate reader (Bio-Tek µQuant, USA). All plasma samples were assayed in duplicate. The concentration of H_2S was calculated against a calibration standard curve of NaHS (3.125–250 µM).

3.2.4.3 Measurement of NO concentration in plasma and corpus cavernosum

In physiological condition, NO is rapidly oxidised into nitrate and nitrite (which constitute stable end products of NO metabolism) and these were measured by the assay protocol. This was then taken as a reflection of endogenous synthesis of NO (Li *et al.*, 2009b). The assay was performed according to the method described by Green and co-workers with some modification (Green *et al.*, 1982). Plasma samples were diluted 5 times with 100 mM phosphate buffer (pH 7.4), while CC homogenates were used undiluted for this experiment.

The diluted plasma/CC homogenate (80 µl) was incubated in 20 µl of enzyme/co-factor mastermix (comprising of 250 µl of 0.1 mM FAD, 250 µl of 2 mM β-NADPH and 500 µl of 2 U/ml nitrate reductase (NR) enzyme) for 30 minutes at 37°C and protected from light. The NR used, assisted with its co-factors β-NADPH and FAD, will reduce nitrate in the samples to nitrite. Griess reagent (100 µl of solution containing an equal volume of 0.2% w/v NED in 5% H_3PO_4 and 2% w/v sulphanilamide in 5% H_3PO_4; the two parts mixed within 12 hours of use and kept chilled) was added and the mixture was incubated for 15 minutes at 37°C. The nitrites in the reaction mixture (including nitrites present in the sample and nitrites derived from nitrates by NR) will react with the Griess reagent to form Griess chromophore (purple azo dye) whose absorbance can be measured with a spectrophotometer (Bio-Tek µQuant) at 540 nm. The nitrite concentration was calculated from a standard curve of $NaNO_2$ (0-87.5 µM).

3.2.5 Experimental protocol to investigate effects of H_2S on expression of targeted mRNAs *in vitro*

Rat CC was first isolated, cut into small strips (approximately 1 mm^3 in dimension) and placed in a 24-well plate in serum-free DMEM. The rat CC was treated as follows: 1) medium control, and 2) NaHS (0.1, 0.5, 1, 2 and 10 mM). The drug was diluted in serum-free DMEM. The tissue was treated with NaHS for 6 hours at 37°C in a CO_2 incubator.

3.2.5.1 Extraction of total RNA from rat corpus cavernosum

About 50-60 mg of rat CC was mixed with 1 ml of Trizol (Invitrogen) and homogenised (Ultra-turrax, IKA® T10 basic) in ice. The tissue homogenate was centrifuged at 12000 g for 10 minutes at 4°C to remove insoluble materials (e.g. proteins, fats, polysaccharides and extracellular materials). This supernatant was then decanted into a fresh RNase-free tube and incubated at room temperature for 5 minutes to allow for the complete dissociation of

nucleoprotein complexes. Then, 0.2 ml of chloroform was added to the homogenate per ml of Trizol used. The tube was tightly capped, shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. The mixture was centrifuged at 12000 g for 15 minutes at 4°C. After centrifugation, the mixture separates into 3 phases: a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The RNA will remain exclusively in the upper aqueous phase. This phase was transferred to another fresh RNAse-free tube. About 0.5 ml of isopropyl alcohol was added (per 1 ml of Trizol used) to precipitate the RNA from the aqueous phase. The mixture was allowed to incubate for 10 minutes at room temperature and then centrifuged at 12000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol (dissolved in diethylpyrocarbonate (DEPC) -treated water). The sample was mixed by vortexing and centrifugation at 7500 g for 5 minutes at 4°C. At the end of the procedure, the RNA pellet was air-dried for 5-10 minutes and dissolved in 100 µl of DEPC-treated water. The RNA solution was incubated at 55°C for 10 minutes to ensure complete dissolution. The RNA obtained was then stored at -70°C until it was used.

Disposable gloves and mask were worn at all times throughout the procedure to prevent introduction of RNase into the sample. Similarly, disposable and filtered pipette tips and RNAse-free tubes were used during the RNA isolation. Laboratory bench tops, glassware/plasticware and equipments used (pipettes) were treated with RNase decontamination solution (RNase Zap®) before the start of the experiment and periodically throughout the procedure. Total RNA isolated was quantified using a nanodrop machine (ND-1000, Nanodrop Technologies) by measuring its absorbance at 260 nm.

Nanodrop was also used to obtain the A_{260}/A_{280} ratio of each RNA sample. This ratio is used to assess the purity of an RNA preparation (RNA samples with A_{260}/A_{280} ratio > 1.8 suggest minimum protein contamination). To assess the integrity of the RNA samples, the samples were run on 1% agarose gel with TBE buffer at 100 V for 30-40 minutes. The presence of

sharp 28S and 18S rRNA bands (with intensity in the ratio of 2:1) and absence of low molecular weight smear would indicate relatively intact RNA samples with little degradation. Only intact RNA samples were used for experiments.

3.2.6 Reverse transcription of RNA to cDNA

The RNA samples were first treated with DNase I to degrade any contaminating genomic DNA template. The DNase I was then heat-inactivated at 75°C for 10 minutes in presence of 4 mM EDTA. The RNA samples (5 µg) was added to RNase-free tube containing 3 µl of 50 µM oligo(dT) primers and 3 µl of 10 mM dNTPs. This RNA/primer mixture was incubated at 65°C for 5 minutes in a thermocycler (ThermoHybaid) and placed on ice. An enzyme mastermix comprising of 12 µl of 25 mM $MgCl_2$, 6 µl of 0.1 M DTT, 3 µl RNase OUT enzyme and 3 µl of Superscript III enzyme was added to the RNA/primer mix according to manufacturer's instruction. The resulting mixture was incubated at 50°C for 50 minutes and at 85°C for 5 minutes in the thermocycler. After the first strand synthesis was completed, 3µl of RNaseH was added to remove the RNA templates. The resulting cDNA samples were stored at -20°C until they were used.

3.2.7 Real Time (Quantitative) RT-PCR

Real time PCR/ qPCR analysis was performed in duplicate or triplicate in a thermal cycler (ABI Prism® 7000 Sequence Detection System, Applied Biosystems, USA) set to the following thermal cycling parameters: *Taq* activation at 95°C for 10 minutes, 50 cycles of denaturation at 95°C for 15 s and combined annealing and extension step at 60°C for 1 minute. The PCR reaction took place in a total volume of 20 µl as follows (Table 3a and 3b):

Components	Volume
cDNA template	2 µl
Forward Primer (5 µM)	1 µl
Reverse Primer (5 µM)	1 µl
SYBR Green Master mix (2X)	10 µl
DEPC-treated H_2O	6 µl
Total :	20 µl

Table 3a. Real time RT-PCR mixture

Genes	Primer Sequence	Expected amplicon size (bp)	GenBank Access Number	Ref
eNOS	*(F)* 5'-CAG CAC CAG ACC ACA GCC CC-3' *(R)* 5'- TCC TGC TGA GCC TGT GCA CT-3'	121	NM_021838.2	(Petersson *et al* 2007)
sGCα1	*(F)* 5'- ACA CAA TAT GCA TCT CCG ATG G-3' *(R)* 5-' GCT CTC TAT ACT CGC TTT GAC CAA-3'	80	NM_017090.2	(Krumenacker *et al* 2001)
sGCβ1	*(F)* 5'- CCC GTG GAA ACT GAT GTC AA-3' *(R)* 5'- CGG GAC CTA GTA GTC ACG CA-3'	129	NM_012769.2	(Krumenacker *et al* 2001)
RhoA	*(F)* 5'-AAG GAC CAG TTC CCA GAG GT-3' *(R)* 5'-TGT CCA GCT GTG TCC CAT AA-3'	110	NM_057132.3	(Corteling *et al* 2007)
Rho-Kinase I (ROCK I)	*(F)* 5'-TTT CGG ATT CAA CTA GTG TTG C-3' *(R)* 5'-TGC AGG CAG AAC CAA CTG-3'	78	NM_031098.1	(Zhang *et al* 2010)
Rho-Kinase II (ROCK II)	*(F)*5'-GCA CAT GTA TGA AAA TGG ATG AAA C-3' *(R)*5'- CAT AAT TTT GCT GTA GGT TCC TAC AAG T-3'	221	NM_013022.1	(Tahara *et al* 2002)
β-Actin	*(F)*5'- GGT CCA CAC CCG CCA CCA GTT-3' *(R)* 5'-ACC CAT ACC CAC CAT CAC ACC CTG-3'	169	NM_031144.2	(Petersson *et al* 2007)

Table 3b. Primer sequences for each gene of interest, including eNOS, sGCα1, sGCβ1, ROCK I, ROCK II and β-Actin. (F) = Forward primer; (R) = Reverse primer

The SYBR Green Master mix contains SYBR Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, Passive Reference, and optimised buffer components. The mixture was loaded into a MicroAmp Optical 96-well reaction plate (Applied Biosystems Cat #4306737) and sealed with an optical adhesive cover (Applied Biosystems Cat # 4311971). No template control (NTC) was run together with the samples in each PCR reaction (NTC contains DEPC-treated H_2O in place of the cDNA template) in triplicate to check for any contamination in the master mix and/or primer dimer formation. The primers for eNOS and β-actin bridge at least 1 intron, thereby minimizing further the chance of any contaminating genomic DNA being amplified in the PCR reaction.

Relative quantitative values (RQ) were calculated based on the difference in threshold cycle number (C_t) between different samples. The fold change in mRNA expression was calculated using the $\Delta\Delta C_t$ method. The measurement of target genes in all samples was normalised to the housekeeping gene β-actin. Data was presented as fold change in expression (as determined by RQ values) in treated groups, relative to control sample.

Since SYBR green non-specifically binds to dsDNA, it is not possible to identify the PCR product from the fluorescent signal alone. To verify that the primers used were specific, the products from the real time PCR were run and visualised on an agarose gel. The size of each amplicon was compared to the expected product size of each PCR reaction as calculated in Table 3b. Primers which produced a single band PCR product at the correct size were considered specific. Dissociation curve (melting curve) analysis was also performed at the end of the real time PCR. Any non-specific product amplified in the PCR reaction and/or any primer dimers would show up in this analysis as a separate peak that is distinct from the desired amplicon peak. Primers which produced only a single distinct peak in the dissociation curve analysis were considered specific. Only primers which produced specific products were chosen for the purpose of experiments.

3.2.8 Experimental protocol to investigate the effects of H_2S on expression of target proteins *in vitro*

To investigate the effects of H_2S on the expression of different proteins *in vitro*, both dose-response and time-response studies were carried out. Rat CC was first isolated, cut into small strips (approximately 1 mm^3 in dimension) and placed in a 24-well plate in serum-free DMEM. The NaHS was dissolved in DMEM and added to each well. Incubation was done at 37°C in a CO_2 incubator.

3.2.8.1 Protein extraction from rat corpus cavernosum tissue

Cell lysis buffer was prepared by mixing 50 µl (0.5%) of protease inhibitor cocktail (Sigma Aldrich, St Louis, MO) to 10 ml of cell lytic mammalian tissue buffer (Sigma Aldrich, St Louis, MO). About 100 mg of the rat CC tissue was homogenised (Ultra-turrax, IKA® T10 basic) in 1 ml of the cell lysis preparation on ice. The homogenate was centrifuged at 14000 g at 4°C for 10 minutes and the supernatant transferred to a fresh tube. The protein samples were stored at -80°C until further use.

3.2.8.2 Isolation of cytoplasmic and total membrane protein

Extraction of cytoplasmic and total membrane protein was done using a membrane protein extraction kit (Promokine, Heidelberg, Germany). About 100 mg of rat CC tissue was homogenised (Ultra-turrax, IKA® T10 basic) in 350 µl of lysis buffer (containing 0.2% protease inhibitor cocktail). The homogenate was then transferred to a 1.5 ml tube and centrifuged at 700 g at 4°C for 10 minutes. The supernatant was decanted to a fresh tube and centrifuged again as above; this process was repeated until no pellet was seen after centrifugation. The resulting supernatant was centrifuged at 10000 g at 4°C for 30 minutes. The supernatant contained cytosolic proteins while the pellet contained total cellular

membrane protein (TMP) - comprising proteins originating from both plasma membrane and cellular organelle membrane. The TMP pellet was dissolved in 50 µl of PBS with 0.5% TritonX-100. The samples were stored at -80°C until analysis. Protein quantification was done using the Bradford assay (Biorad, CA, USA) and by measuring its absorbance at 280 nm using a spectrophotometer (ND-1000, Nanodrop Technologies).

3.2.8.3 Western blot

About 15-30 µg of protein samples were mixed with the sample buffer (Laemmli buffer containing 5% β-mercaptoethanol, which was freshly prepared on the day of sample run), boiled at 96°C for 5 minutes and loaded into 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed at 80 V for 20-25 minutes (through the stacking gel) and then at 120 V for 75-85 minutes (through the resolving gel). The proteins were subsequently transferred from the gels to PVDF membranes with the following transfer condition: 100 V for 90-100 minutes at 4°C with constant stirring. The membranes were then blocked with 5% blocking grade non fat dry milk in PBS containing 0.1% Tween20 (PBST) at room temperatures for 60 minutes. After blocking, the PVDF membranes were incubated with their respective primary antibodies as listed in Table 4. Pan actin (which represented all isoforms of/total actin) was used as loading control. The primary antibodies were incubated at 4°C overnight in an orbital shaker (IKA® KS260basic, Germany). The membranes were then washed in PBST and incubated with their respective horseradish peroxidase (HRP)-conjugated secondary antibodies (please refer to Table 4) for 1.5 hours. The membranes were then washed again in PBST. Protein bands were visualised by the addition of enhanced chemiluminescent (ECL) substrates for HRP (SuperSignal® West Pico Chemiluminescent Substrate, Pierce) and captured by exposure to film (Thermoscientific Pierce Cat # 0034089). Protein was quantified by measuring band area and intensities in the film using Image J software. The results were expressed as fold difference in protein expression of treated groups to that of control.

Protein of interest	Manufacturer	Primary (1°) antibody dilution	Nature of 1° Antibody	Secondary antibody dilution	Protein size (kDa)	Reference
sGCα1	Sigma (Cat #G4280)	1:1000	Rabbit; polyclonal	1:1000	~80	Nangle and Keast, 2007
sGCβ1	Sigma (Cat #G4530)	1: 1000	Rabbit; polyclonal	1:1000	~70	Nangle and Keast, 2007
RhoA	Santacruz Biotechnology (Cat #26C4 (sc418))	1: 200	Mouse; monoclonal	1:1000	~24	Bivalacqua *et al*., 2004; Wang *et al*., 2002
ROCK II	BD Transduction (Cat #611136)	1: 300	Mouse; monoclonal	1:1000	~180	Bivalacqua *et al*., 2004; Wang *et al*., 2002; Jin *et al*., 2006
Pan actin	Millipore (Cat #MAB1501R)	1: 5000	Mouse; monoclonal	1: 10000	~42	Poling *et al*., 2011

Table 4. Antibody information (primary and secondary) and the conditions used in western blot for sGCα1, sGCβ1, RhoA and ROCK II. Pan actin was used as loading control.

3.2.9 Experimental protocol to investigate the involvement of testosterone in H_2S' effects

Age-matched rats were randomly assigned to 6 groups (n=7 per group) as follows: 1) normal control, 2) normal NaHS (0.8 mg/kg, ip, daily), 3) normal testosterone (10 mg/kg, im, twice weekly), 4) castrated control, 5) castrated NaHS (0.8 mg/kg, ip, daily), 6) castrated testosterone (10 mg/kg, im, twice weekly). The NaHS was dissolved in distilled H_2O just prior to administration. All treatments were continued for 10 weeks. At the end of the treatment, changes in the rats' intracavernosal pressure to electrical stimulation were measured (as in section 3.2.3.1). The rats were then euthanised and their plasma collected and stored at -80°C for measurement of concentration of testosterone, H_2S, and NO.

3.2.9.1 Castration procedure in rat model

Total (closed) orchiectomy of the rat was performed under isoflurane anesthesia by standard procedure (Dulisch, 1976). Throughout the surgical procedure, the rat was placed in a heating pad set at 37°C to minimise heat loss, and its heart rate and oxygen level in the blood were closely monitored with a pulse oxymeter. The hair over the inguinal area was removed and the skin area was disinfected with 70% alcohol and iodine solution. The testicle was massaged out of the abdominal cavity into the scrotum and held in position. The inguinal skin was carefully incised and the testicle was pulled out through this small incision without opening the tunica vaginalis. The spermatic cord and blood vessels were identified and clamped with a mosquito hemostat. They were then ligated proximal to the clamp with an absorbable suture and excised. After the haemostat was released, the stump was held with a pair of forceps distal to the ligature and visual inspection was done to ensure absence of hemorrhage. The skin incision was finally closed with non-absorbable suture. The procedure was repeated on the opposite side to remove the other testis. Antibiotic (Baytril; 5 mg/kg) and painkiller (Carprofen; 5 mg/kg) were administered intramuscularly at the end of the surgery and continued for 5 consecutive days post-surgery. Broad spectrum antibiotic powder (Baneocin) was also applied topically daily until the wound healed completely. A recovery period of 4 weeks was allowed before starting the study.

3.2.9.2 Measurement of testosterone concentration

Testosterone concentration was measured using Immulite1000 analyzer (Siemens, USA) (Friedrich *et al.,* 2008). The process was an automated solid-phase competitive chemiluminescent enzyme immunoassay. This assay was based on competitive binding between labelled (testosterone conjugated with alkaline phosphatase; this is referred to as ‘reagent wedge’ in the kit) and non-labelled free testosterone in the samples for a limited testosterone antibody binding site (in the form of solid phase ‘test unit’ which contains a

polystyrene bead coated with polyclonal rabbit anti testosterone antibody). The analyzer was calibrated using adjustors which are vials of 4 ml each of testosterone in high and low concentration. The plasma sample (100 µl) was placed in the cup holder, which was loaded into the analyzer. The wedge and chemiluminescent substrate were also loaded inside the analyzer before running the test. The analyzer would introduce the sample along with the labelled testosterone into the reaction tube containing the bead and incubate them for 60 minutes at 37°C with intermittent agitation. The unbound labelled testosterone and unbound samples were removed by centrifugal wash. The analyzer would then add the chemiluminescent substrate and let the reaction tube incubate for 5 minutes at 37°C. The substrate undergoes hydrolysis in the presence of alkaline phosphatase and emits light. This light signal/photon count would be measured by the photomultiplier tube (PTM) and converted to analyte concentrations (doses) using stored master curves. The amount of bound labelled testosterone was determined and was inversely proportional to the amount of non-labelled/free testosterone present in the samples. The overall concentration of the testosterone in the sample was then calculated and generated by the analyzer.

3.2.10 Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Statistical evaluation was done using *t*-test for significant differences in mean values for a variety of variables between pairs of groups. One-tailed t-test was used when the hypothesis was tested in one direction (Ho: $\mu 1 < \mu 2$ or Ho: $\mu 1 > \mu 2$) whereas two-tailed t-test was used for testing the hypothesis in both directions (Ho: $\mu 1 \neq \mu 2$). One-way ANOVA followed by Bon ferroni's test was used for multiple comparisons across groups. All statistical calculations were done using SPSS software for windows. P-values < 0.05 were considered statistically significant.

4. RESULTS

4.1 Effects of treatments *in vivo*

The average body weight of the rats before treatment was in the range of 328.5 ± 5.4 g – 391.4 ± 4.5 g. The percentage body weight change at the end of the treatment period (10 weeks) was 178%, 136%, 153%, 132% and 127% for the control, sildenafil, NaHS, L-NAME and PAG group respectively. This percentage change was significantly less ($p < 0.05$) in all the treatment groups compared to control.

Rat erectile function was assessed at the end of the 10-week period by evaluating the changes in intracavernosal pressure to electrical stimulation. In the physiological system, without full penile tumescence and the blocking of venous return, ICP will not elevate maximally. Hence, any increase in ICP is an indicator of the entry of arterial blood into the CC and the appropriate venous blockage, taken as an equivalent of physiological erection. ICP has been found to be a suitable experimental index for the evaluation of penile erection in the rat model (Chen *et al*., 1992). For the net change in ICP, the peak reading during electrical stimulation was compared as a function of the basal reading before stimulation. For each animal whose ICP was measured, its respective MAP was also measured. In the control group, the MAP was 116 ± 4.7 mmHg. There were no significant differences in MAP in sildenafil and PAG-treated group compared to control (Figure 4.1). NaHS however exhibited marked hypotensive effect, decreasing the MAP to 95.7 ± 6.8 mmHg while L-NAME pre-treatment caused an increase in the blood pressure to 138.2 ± 9.6 mmHg.

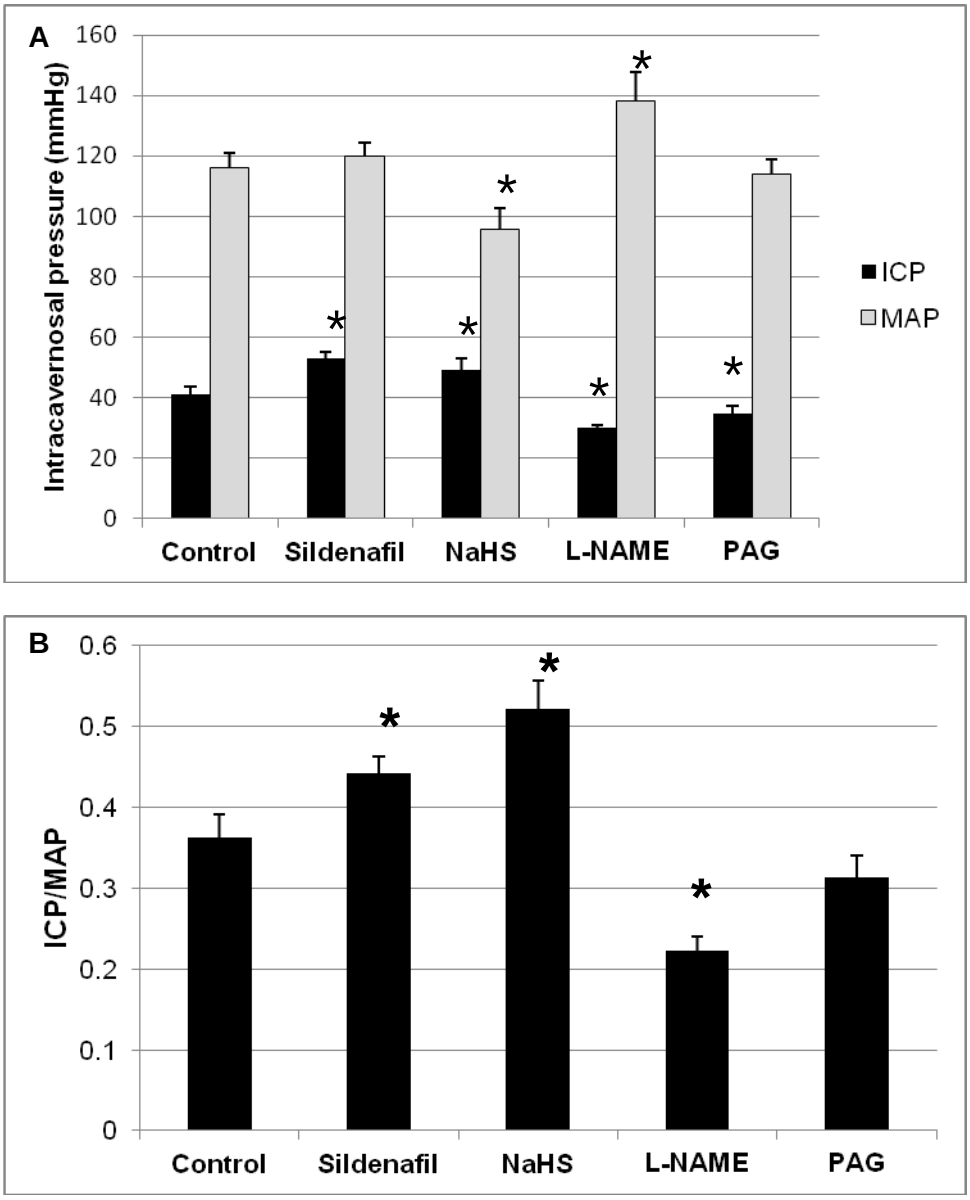


Figure 4.1 Effects of treatments on magnitude of erectile response to electrical stimulation. Measurement was done *in vivo* on ketamine/xylazine-anaesthetised Sprague Dawley (SD) rats at the end of a 10-week long treatment period. (A) Changes in ICP and mean arterial pressure (MAP); (B) changes in ICP after normalizing it to the mean arterial pressure (ICP/MAP ratio). Each bar represents the mean ± SEM of measurements from 7 animals. * P-value < 0.05 (increase/decrease). L-NAME = Nω-Nitro-L-arginine methyl ester hydrochloride; PAG = DL-propargylglycine; NaHS = sodium hydrosulphide

The NaHS treatment was found to augment the rise in ICP to electrical stimulation at the stipulated parameter, as evidenced from the higher ICP/MAP ratio of 0.52 ± 0.03 compared to 0.36 ± 0.02 in the control group ($P < 0.05$). However, PAG did not appear to decrease the magnitude of erectile response significantly (ICP/MAP of 0.31 ± 0.02 compared to 0.36 ±

0.02 in control; $P > 0.05$) after normalizing the ICP to MAP even though PAG caused reduction in ICP alone (Figure 4.1). As expected, sildenafil pre-treatment improved the magnitude of erectile response to 0.44 ± 0.01 in marked contrast to L-NAME treatment which significantly reduced erectile response, compared to control (ICP/MAP of to 0.22 ± 0.01 and 0.36 ± 0.02 respectively; $P < 0.05$). The gross increase in ICP did not approach MAP in this experimental setting.

4.2 Effects of treatments on NO level in plasma and corpus cavernosum *in vivo*

It was observed that NaHS had a systemic effect on the NO/cGMP pathway when administered in the long term, as indicated by the measurement of plasma NO. Treatment with NaHS significantly ($P < 0.05$) increased the plasma NO to 23.0 ± 1.15 µM; an elevation of approximately 47% compared to the control group (15.7 ± 0.88 µM) (Figure 4.2). This was comparable to sildenafil pre-treatment, which increased the plasma NO by 58% (15.7 ± 0.88 and 24.9 ± 2.24 µM in the control and sildenafil group respectively). Conversely, PAG reduced the plasma NO by 22%. This reduction was significant ($P < 0.05$) and comparable to L-NAME which lowered the plasma NO by 26%.

A similar effect of NaHS and sildenafil was also observed locally; NaHS treatment increased the NO concentration in the cavernosum dramatically (from 3.6 ± 0.36 to 6.3 ± 0.85 µmol/g protein; a 73.4% increase) whereas sildenafil increased the corpus cavernosum NO level by 38% (from 3.6 ± 0.36 to 5.0 ± 0.70 µmol/g). It was observed that L-NAME reduced the NO level in CC by 9% although this decrease was not statistically significant. Interestingly, PAG increased the NO level by 47%.

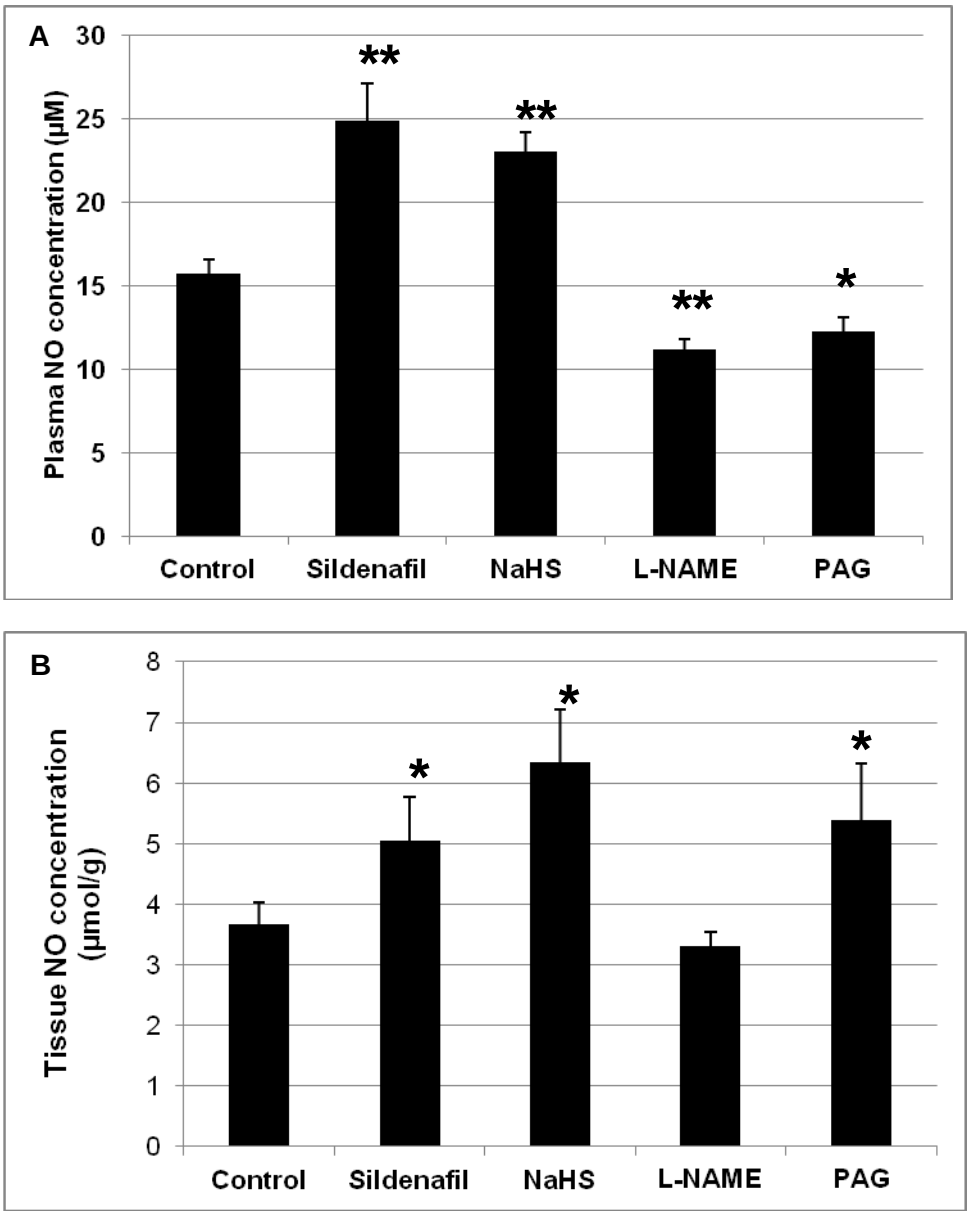
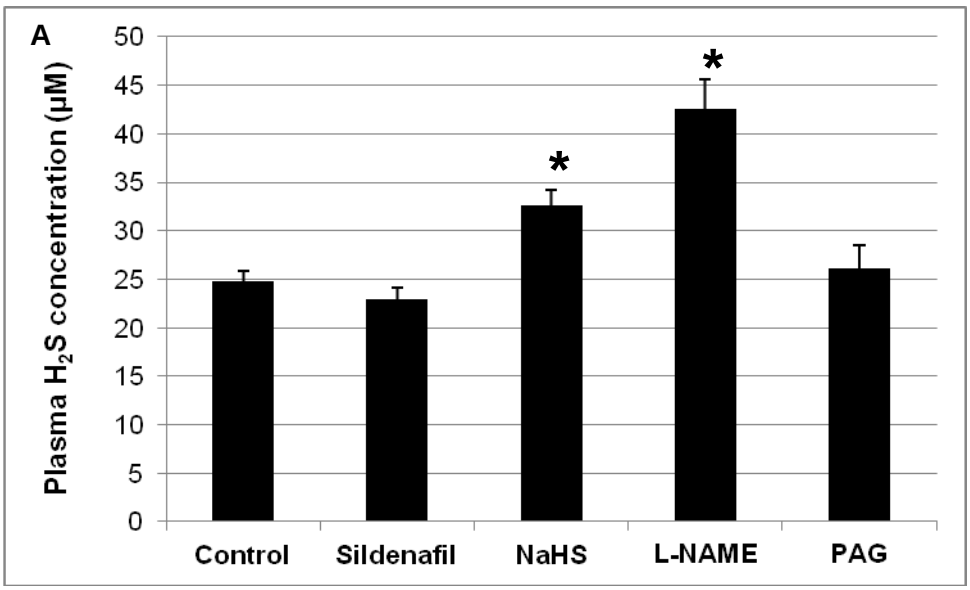


Figure 4.2 Effects of chronic *in vivo* treatments of sildenafil, NaHS, L-NAME and PAG on nitric oxide concentration in (A) plasma and (B) corpus cavernosum. Each bar represents the mean ± SEM of measurements from 7 animals. * P-value < 0.05; ** P value < 0.005 (increase/decrease). L-NAME = Nω-Nitro-L-arginine methyl ester hydrochloride; PAG = DL-propargylglycine; NaHS = sodium hydrosulphide

4.3 Effects of treatments on H_2S level in plasma and H_2S production in corpus cavernosum *in vivo*

In vivo treatment with NaHS not only elevated H_2S concentration in the plasma (from 24.7 ± 1.07 to 32.6 ± 1.63 µM; an approximately 31% increase), but it also increased the H_2S-producing capacity in CC from the basal level of 4.4 ± 0.33 µmol/g/hr in the control group to 7.3 ± 0.75 µmol/g/hr; this represents a 64% increase in the tissue's H_2S-synthesizing capacity (Figure 4.3). It was observed that PAG treatment *in vivo* had no effects on both H_2S level in plasma as well as H_2S production in CC. Rats treated with sildenafil also did not show any changes in the plasma H_2S level and H_2S production in CC. On the other hand, L-NAME-treated group exhibited a significantly elevated plasma H_2S concentration of 42.5 ± 3.06 µM compared to control (24.7 ± 1.07 µM); 71% higher level compared to control. However L-NAME did not seem to affect H_2S biosynthesis in the CC.



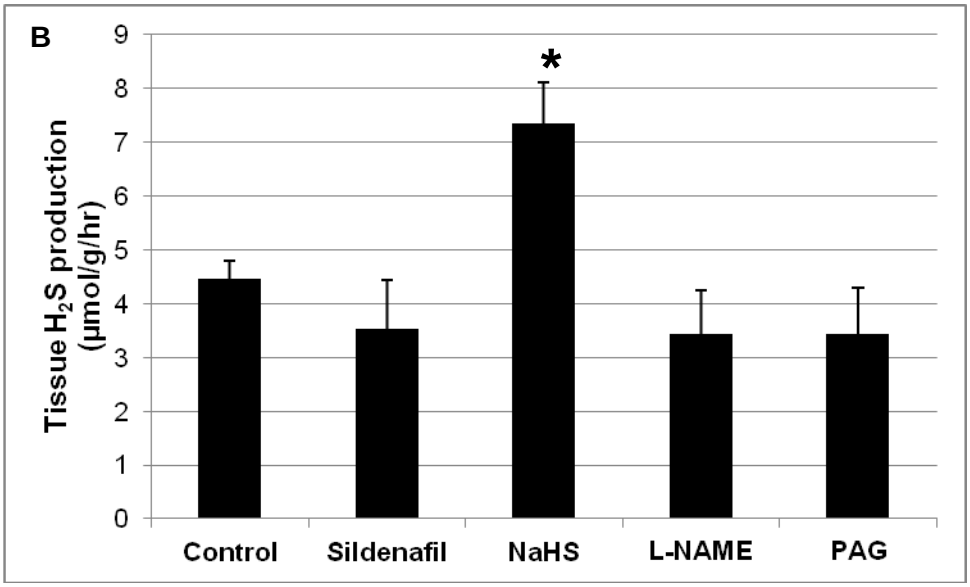


Figure 4.3 Effects of chronic *in vivo* treatments of sildenafil, NaHS, L-NAME and PAG on (A) hydrogen sulphide concentration in plasma and (B) hydrogen sulphide production in corpus cavernosum. Each bar represents the mean ± SEM of measurements from 7 animals. * P-value < 0.001 (increase). L-NAME = Nω-Nitro-L-arginine methyl ester hydrochloride; PAG = DL-propargylglycine; NaHS = sodium hydrosulphide

4.4 Effects of NaHS on cGMP and cAMP level *in vitro*

Cyclic GMP level was measured from the lysate of rat CC primary culture *in vitro*. It was observed that NaHS caused a dose dependent elevation in the level of cGMP (Figure 4.4). The concentration of cGMP was raised from 0.11 ± 0.01 pmol/mg in the medium control group to as high as 0.30 ± 0.06 pmol/mg with 10 mM of NaHS. Unlike with cGMP, the effect of NaHS on cAMP level appeared to be more dose-sensitive with a dose of 1 mM significantly increasing the cAMP level from 5.0 ± 0.33 in the medium control group to 7.0 ± 0.61 pmol/mg. Despite the trend, the effect on cAMP level observed with other doses of NaHS used in the study, failed to reach statistical significance (Figure 4.5). For the treatment group, NaHS dose higher than 10 mM was excluded as it was supraphysiological and therefore unlikely to have any physiological implication.

The cGMP level in the CC was about 50 fold lower than cAMP level per mg protein but despite this low basal (control) level, NaHS was able to significantly increase the cGMP concentration. In terms of percentage of change, the maximum increase in cGMP level that was induced by NaHS was more dramatic (171.8 % change) than cAMP (38.7 %).

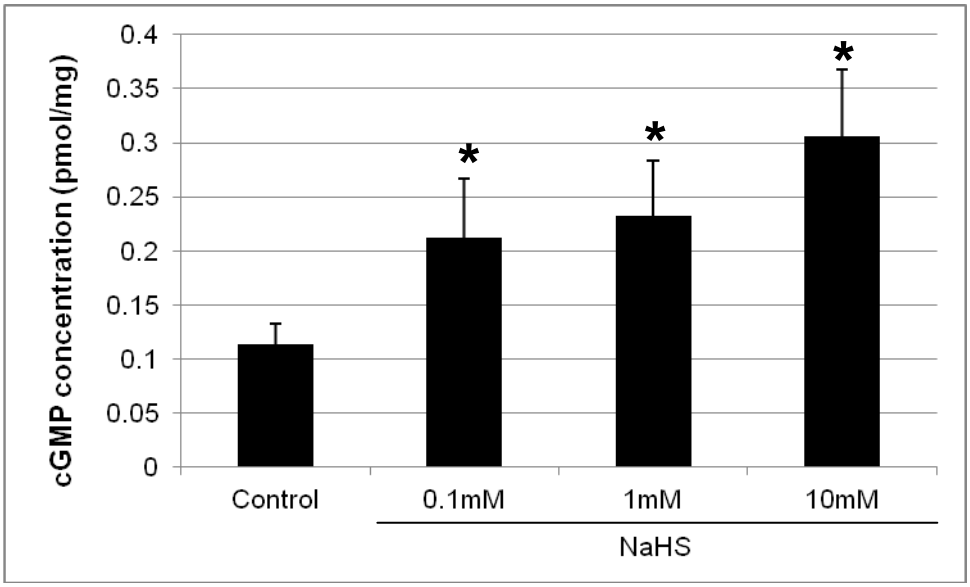


Figure 4.4 Effects of 30 minutes incubation of NaHS at indicated dosage on cGMP concentration in primary culture of rat corpus cavernosum at passage 1-3. Data shown are the mean ± SEM (n= 10). * P-value < 0.05 (increase).

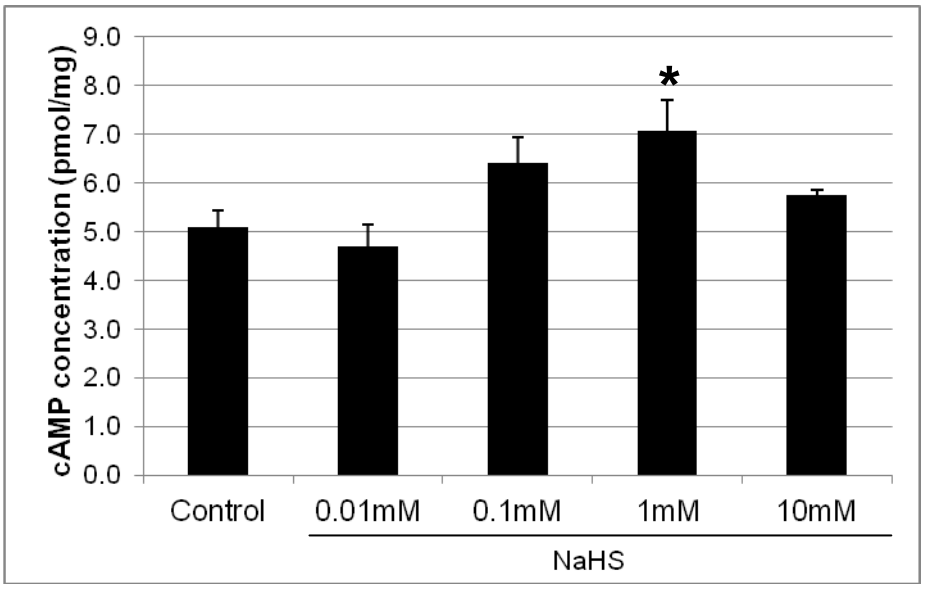


Figure 4.5 Effects of 30 minutes incubation of NaHS at indicated dosage on cAMP concentration in primary culture of rat corpus cavernosum at passage 1-3. Data shown are the mean ± SEM (n= 5-6). * P-value < 0.05 (increase).

4.5 RNA samples

The purity and integrity of the total RNA extracted from the CC were first determined before reverse transcription was performed. The RNA samples were still relatively intact after the Trizol extraction procedure (result not shown). Some degradation was evident as low molecular weight smear but the 28S and 18S rRNA bands were visible and still relatively sharp. All the RNA samples were found to have A_{260}/A_{280} ratio of 1.99-2.00, indicating little/ no protein contamination.

4.6 Gene expression of eNOS

The eNOS and β-Actin primers produced specific products. Only a single band was observed for both eNOS and β-Actin, at 121 bp and 169 bp respectively which was the expected PCR product size. In both cases, the NTC showed little/no amplification and the dissociation curve showed only one peak, indicating that the primers were relatively specific. β-Actin was used as internal control throughout our study.

As mentioned in Section 3.1.1, NaHS was dissolved in DMEM. However, comparison of eNOS mRNA expression between non-treated CC tissue and CC treated with medium revealed that DMEM could affect eNOS expression in the CC tissue samples (result not shown). Therefore, medium control (at the longest incubation period used i.e. 6 hours) was used for comparison in this study. It was shown that NaHS treatment significantly increased the eNOS mRNA level (by almost four fold), when compared to the medium control. This effect of NaHS appeared to be time dependent, with the increase being most prominent at 6 hours (Figure 4.6). The number of experiments that could be carried out was limited by the amount of tissue and/or animals available.

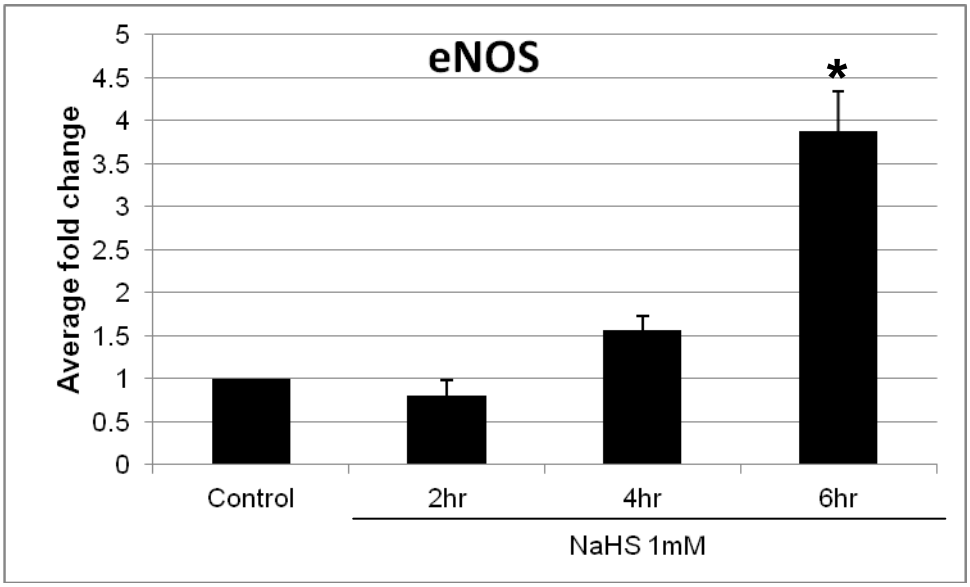


Figure 4.6 Relative expression of eNOS mRNA in rat CC after NaHS treatment at different time points as assessed by real time PCR. Results, normalised to β-actin mRNA, are expressed relative to the medium control taken as 1. Data shown are the mean ± SEM (n=3). * $P < 0.05$ (increase).

4.7 Gene and protein expression of sGCα1 and sGCβ1

The sGCα1 and sGCβ1 primers both produced specific PCR products. Only one band was observed for both sGCα1 and sGCβ1 at approximately 80 bp and 129 bp respectively, which corresponded to the expected amplicon size (results not shown). Dissociation curve analysis on the real time PCR product revealed a single peak when these primers were used, confirming that only one specific product was formed. NTC also showed no amplifications. These observations suggest that the primers used here were specific enough for SYBR-based qPCR.

Generally, NaHS caused a dose dependent increase in the expression of sGCα1 mRNA *in vitro*. This increase was most apparent at 10 mM dose with more than two fold increase in the expression of sGCα1 mRNA (Figure 4.7).

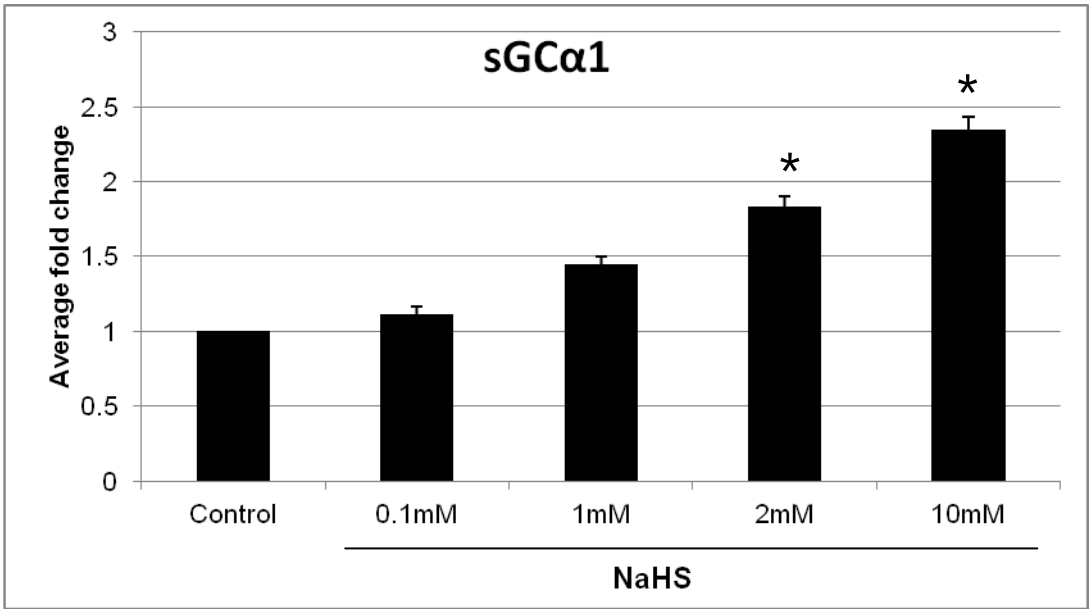
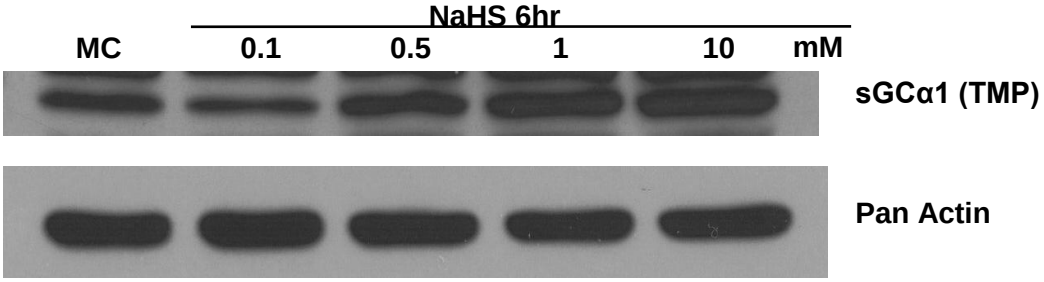


Figure 4.7 Relative expression of sGCα1 mRNA in rat CC as assessed by real time PCR. Effects of different doses (indicated on the x-axis) of NaHS when incubated for 6 hours. Results, normalised to β-actin mRNA, are expressed relative to the medium control taken as 1. Data shown are the mean ± SEM (n=4). * $P < 0.005$ (increase).

The protein expression of sGCα1 is shown in Figure 4.8a-b. It was observed that the trend of sGCα1 protein expression did not really correspond to its mRNA expression. NaHS increased the protein expression of sGCα1 in the cellular membrane (to 1.3 fold compared to control) but decreased its expression in the cytosol (up to 0.48 of the control value).



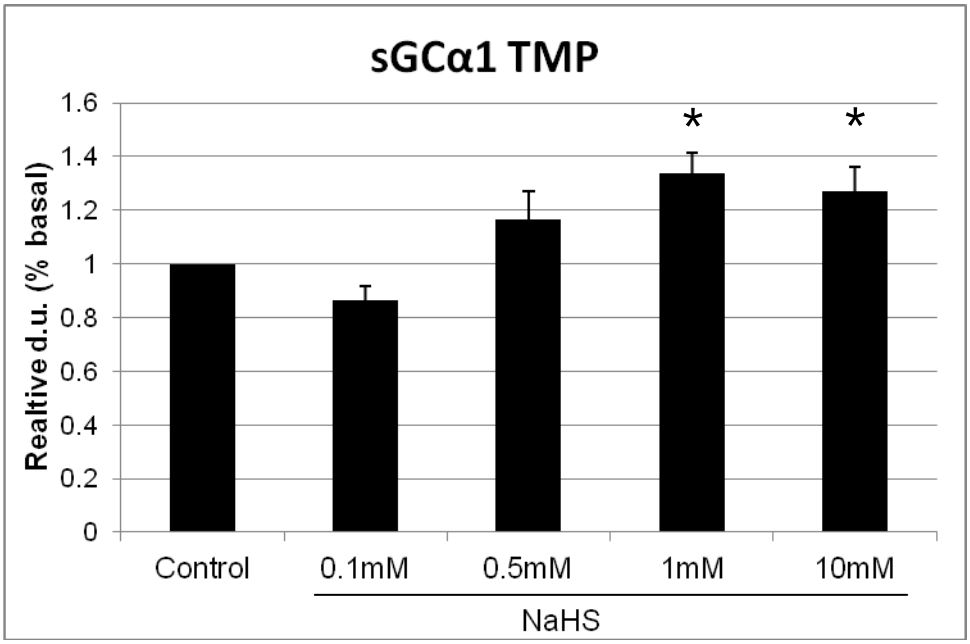
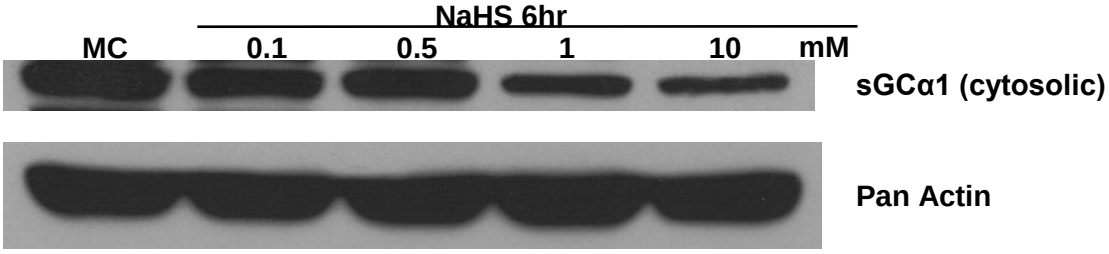


Figure 4.8a sGCα1 protein expression in rat corpus cavernosum (TMP) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=4). * P < 0.05 (increase).



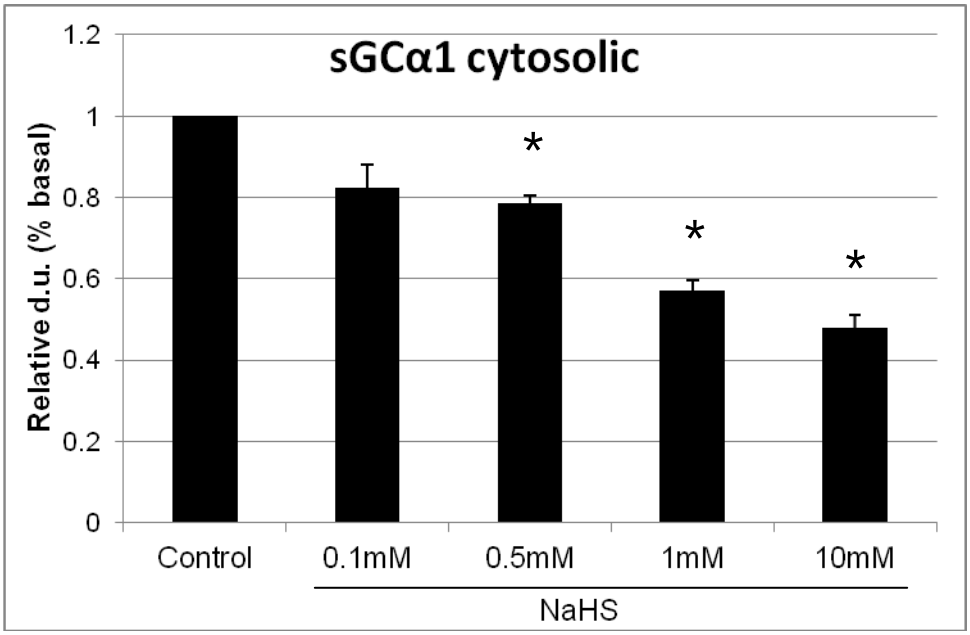


Figure 4.8b sGCα1 protein expression in rat corpus cavernosum (cytosolic fraction) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=4-5). * $P < 0.005$ (decrease).

Similar to its effect on sGCα1, NaHS also increased the expression of sGCβ1 mRNA (Figure 4.9). The increase in mRNA expression was more prominent for sGCβ1 than sGCα1 for the same dose of NaHS used; in particular, 10 mM of NaHS increased the sGCβ1 mRNA expression in the CC by five fold whereas the same dose of NaHS only increased the sGCα1 mRNA expression by 2.3 fold.

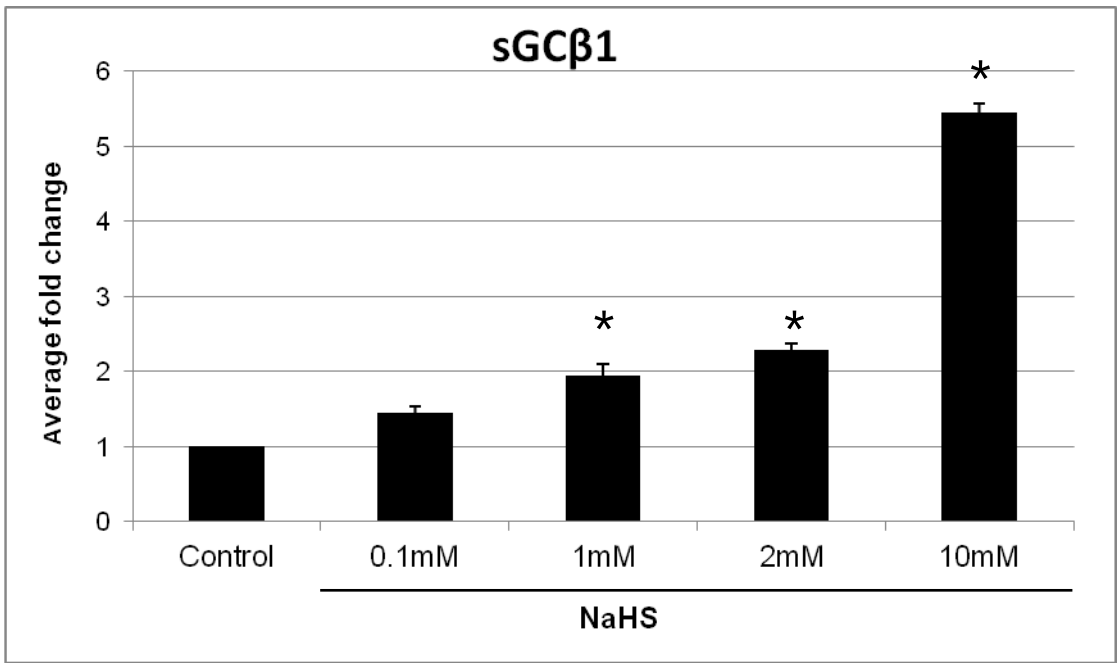


Figure 4.9 Relative expression of sGCβ1 mRNA in rat CC as assessed by real time PCR. Effects of different doses (indicated on the x-axis) of NaHS when incubated for 6 hours. Results, normalised to β-actin mRNA, are expressed relative to the medium control taken as 1. Data shown are the mean ± SEM (n=5). * P < 0.005 (increase).

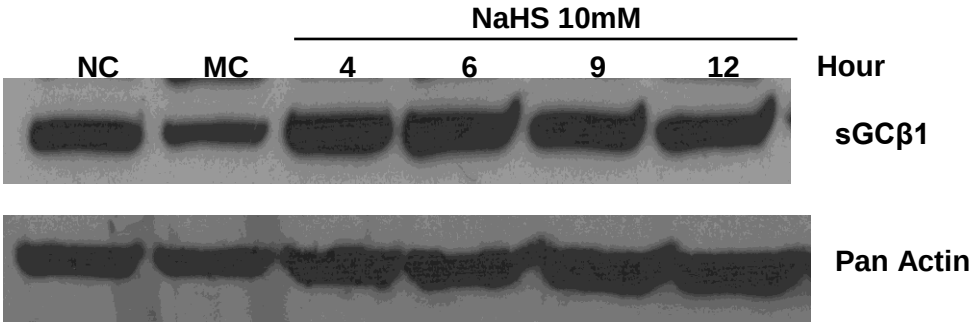
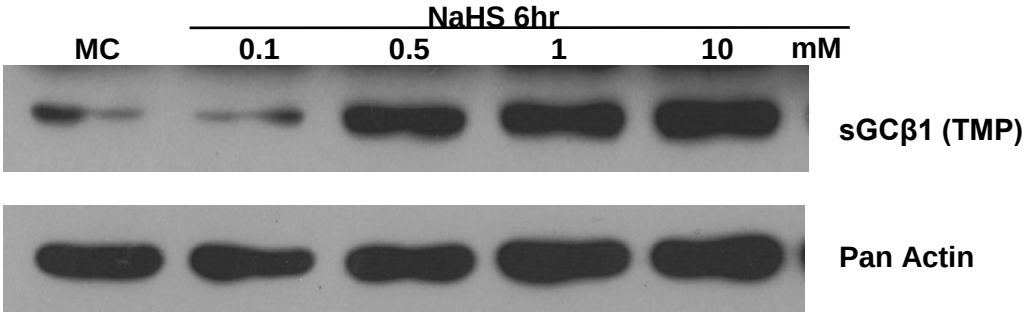


Figure 4.10 Temporal expression of sGCβ1 protein in rat corpus cavernosum (total tissue lysate). Rat CC were treated with 10 mM of NaHS with different incubation period. NC= no treatment control; MC= medium control.

Study of the temporal expression of sGCβ1 at the protein level using western blot revealed that DMEM inhibited the expression of sGCβ1 protein (Figure 4.10) therefore subsequent studies on sGCβ1 expression used medium control (MC) for comparison. Treatment with 10 mM of NaHS significantly increased the sGCβ1 protein expression (compared to MC as baseline) at all the time points used. This result was consistent with the qPCR data since at this dose NaHS also increased the sGCβ1 mRNA level considerably.

Generally, NaHS increased the expression of both membrane-associated and cytosolic sGCβ1 protein in a dose dependent manner (Figure 4.11). Noticeably, only NaHS in the higher mM range (1-10 mM) significantly increased the protein expression of sGCβ1. At this dose range, approximately three-fold increase in sGCβ1 protein was observed. The trend of the sGCβ1 protein expression largely corresponded to that of its mRNA. Temporal expression study (Figure 4.10) showed that incubation of CC with NaHS for up to 12 hours still resulted in significant increase in the sGCβ1 protein expression, suggesting that the up-regulation of sGCβ1 protein expression by H_2S was likely to be sustained i.e. not a transient event.



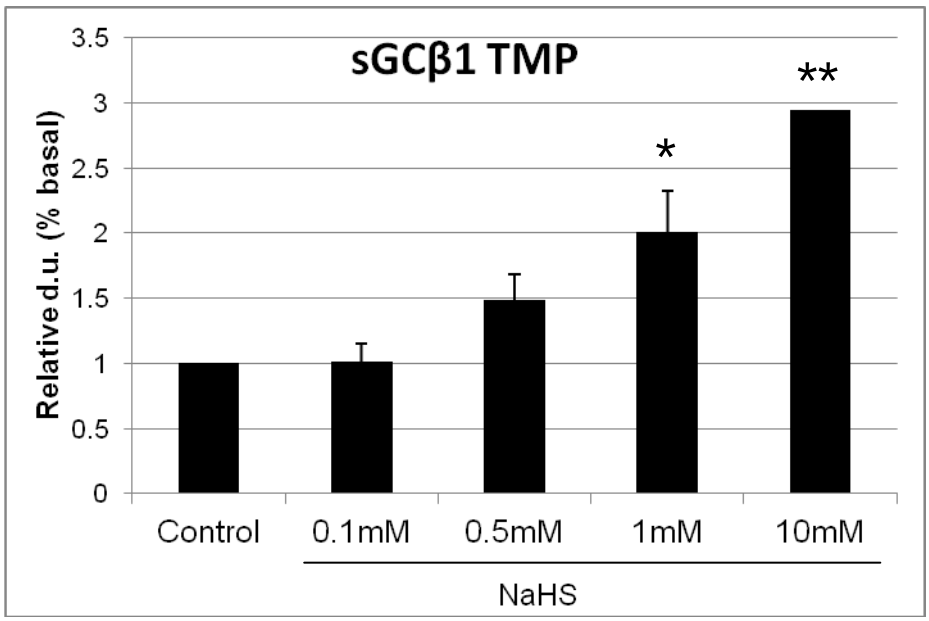


Figure 4.11a sGCβ1 protein expression in rat corpus cavernosum (TMP) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=3-7). * P < 0.01; **P < 0.001 (increase).

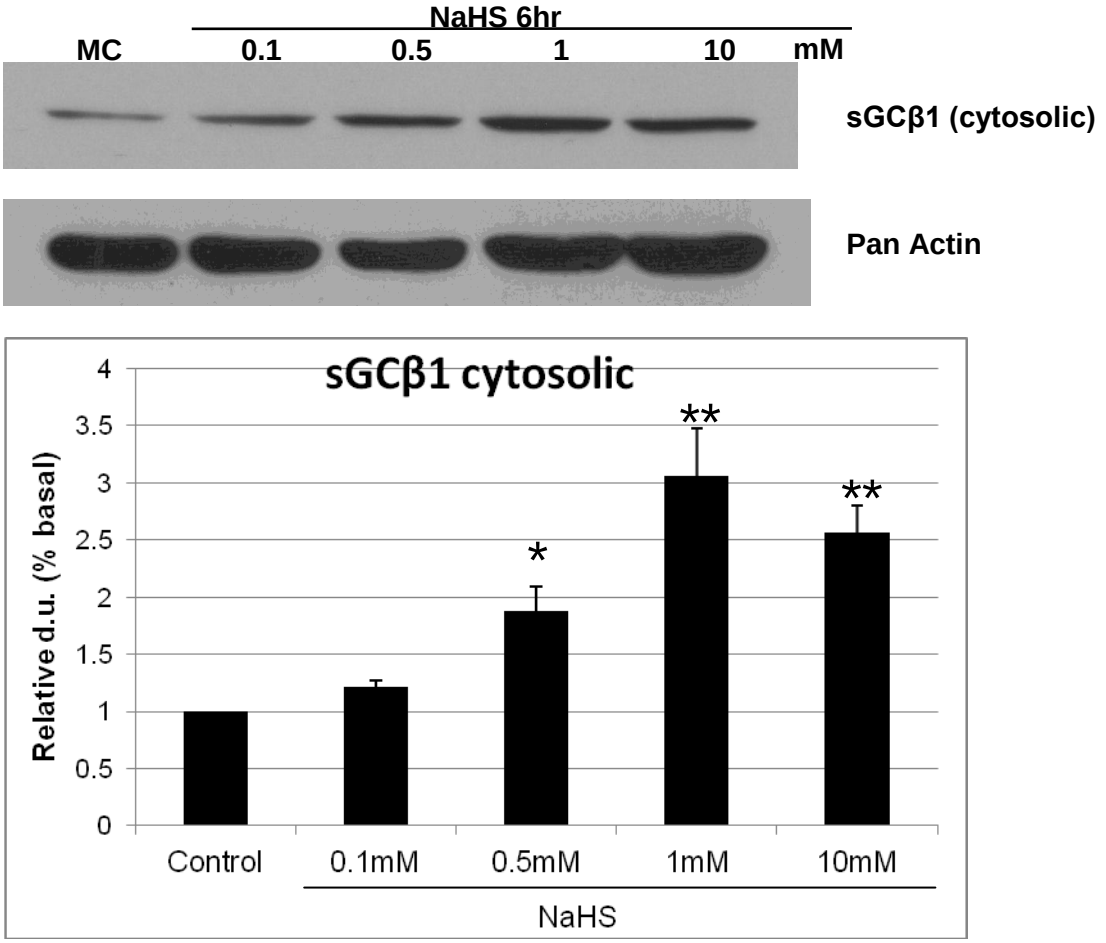


Figure 4.11b sGCβ1 protein expression in rat corpus cavernosum (cytosolic fraction) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=3-5). * P < 0.01; **P < 0.001 (increase).

4.8 RhoA/Rho-Kinase pathway

4.8.1 Gene expression of RhoA, ROCK I and ROCK II

The RhoA, ROCK I and ROCK II primers produced a single specific band/product during the real time PCR reaction. RhoA, ROCK I and ROCK II generated amplicons at approximately 110 bp, 78 bp and 221 bp respectively. All of them corresponded to the expected PCR product size (please refer to Table 3b). The NTC in all the reactions showed no amplification.

Dissociation curve analysis on the real time PCR product revealed a single peak when these primers were used, confirming that only one specific product was formed (not shown). NTC also showed no amplifications. As mentioned earlier, these observations suggest that the primers used here were specific enough for SYBR-based qPCR.

As in the case with sGCβ1 (Figure 4.10), the medium was shown to affect the RhoA mRNA expression as incubation of CC with DMEM lowered the RhoA mRNA level significantly (result not shown). However, it was observed that when compared to medium control, NaHS did not affect the mRNA expression of RhoA *in vitro* in all the doses (0.1-10 mM) used (Figure 4.12).

Very little/no ROCK I mRNA was detected by real time PCR. Some samples showed no amplification at all while some showed amplification after approximately 36 amplification cycles (result not shown), only when the amount of cDNA template was increased. The PCR amplification step had to be prolonged considerably (to > 50 cycles) before a PCR band could be visualised faintly on agarose gel. This result demonstrated that ROCK I mRNA was absent/present in low quantity in the corpus cavernosum, suggesting that its role in erectile response is likely to be limited. On the other hand, ROCK II mRNA could be detected relatively easily in CC. Generally, NaHS had no effect on the ROCK II mRNA expression (Figure 4.13).

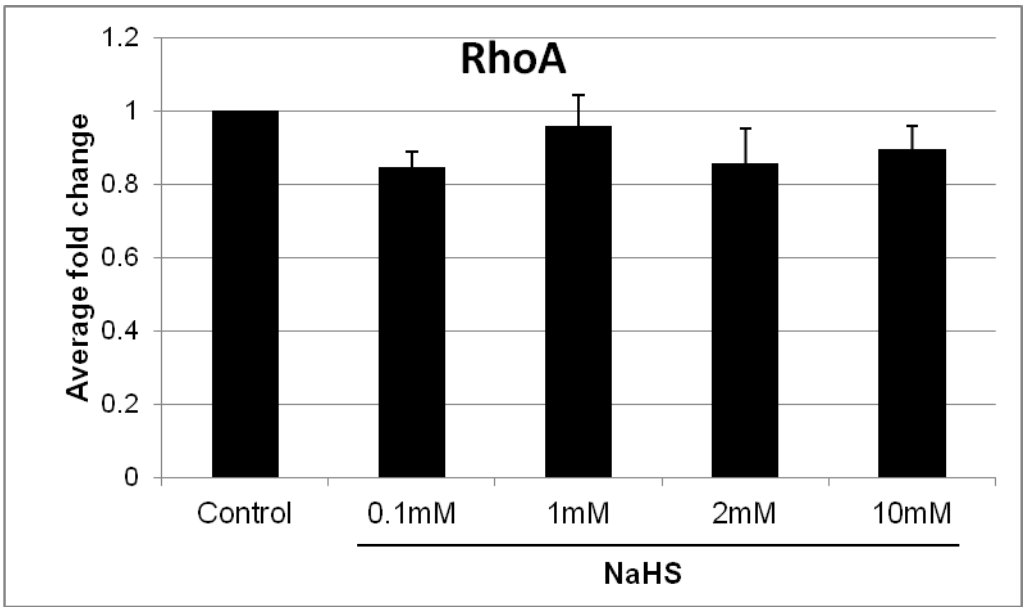


Figure 4.12 Relative expression of RhoA mRNA in rat CC as assessed by real time PCR. Effects of different doses (indicated on the x-axis) of NaHS when incubated for 6 hours. Results, normalised to β-actin mRNA, are expressed relative to the medium control taken as 1. Data shown are the mean ± SEM (n=3).

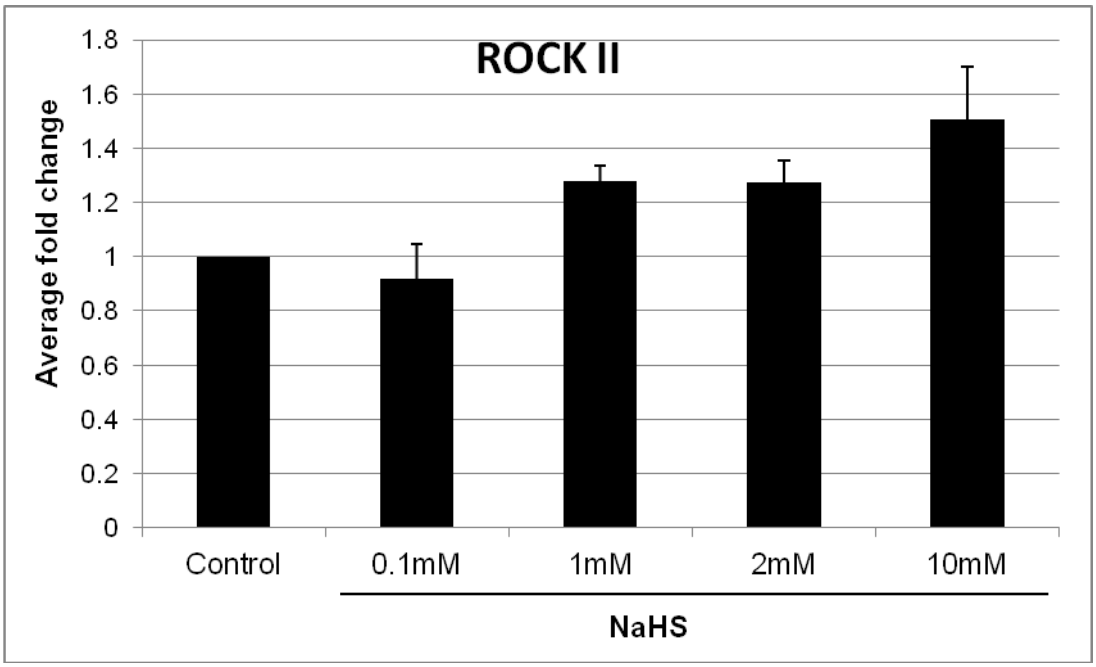


Figure 4.13 Relative expression of ROCK II mRNA in rat CC as assessed by real time PCR. Effects of different doses (indicated on the x-axis) of NaHS when incubated for 6 hours. Results, normalised to β-actin mRNA, are expressed relative to the medium control taken as 1. Data shown are the mean ± SEM (n=4-6).

4.8.2 Protein expression of RhoA and ROCK II

It was observed that NaHS significantly inhibited the expression of RhoA protein, both in the cytosol as well as plasma membrane (Figure 4.14a-b). Compared to control, lower RhoA protein level was present in the plasma membrane of CC treated with 0.5-1 mM of NaHS. At the relatively high dose of 10 mM, NaHS appeared to have opposite effect; it increased the RhoA protein expression in the plasma membrane. The response of the CC to NaHS did not appear to be graded; 0.5 and 1 mM of NaHS resulted in similar degree of inhibition of membrane-bound RhoA protein expression (approximately 40% reduction compared to control).

It was also observed that NaHS lowered the expression of RhoA protein in the cytosol but this only occurred at the concentration of 0.5 mM. The results suggest that there exists a specific therapeutical window where H_2S can inhibit the RhoA pathway and this window may be exploited for future development of H_2S-based drugs to treat ED. Generally, NaHS seemed to affect RhoA only at translational but not transcriptional level since its effect on RhoA mRNA was distinctly marked compared to its effect on RhoA protein expression.

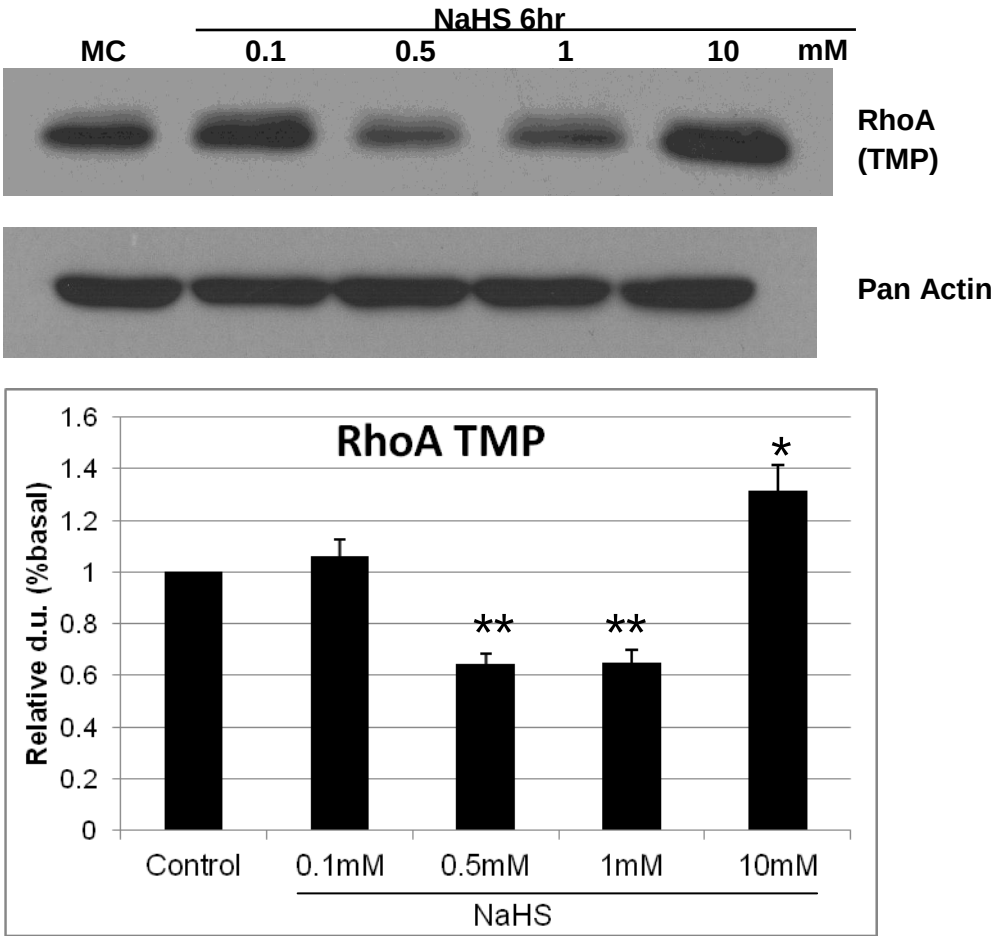
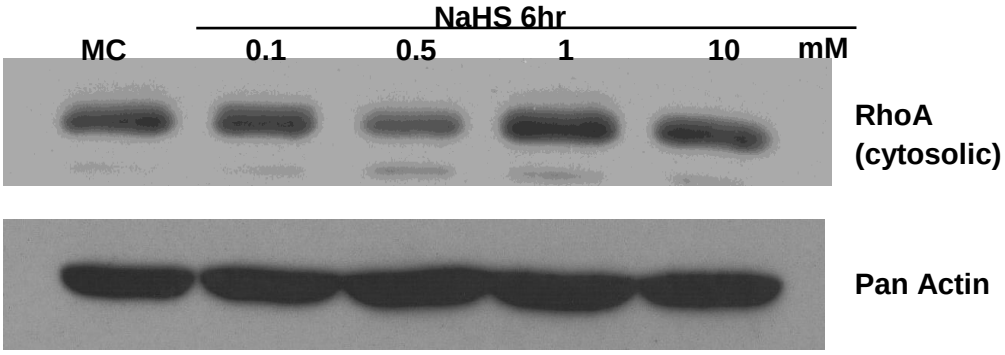


Figure 4.14a RhoA protein expression in rat corpus cavernosum (TMP) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=4-6). * $P < 0.05$ (increase); ** $P < 0.005$ (decrease).



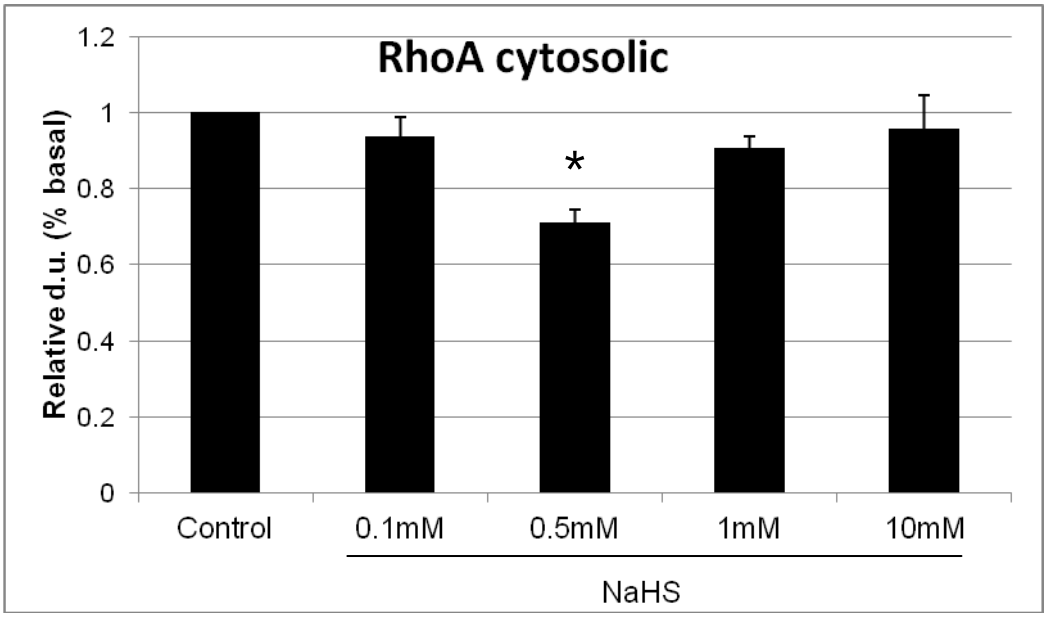
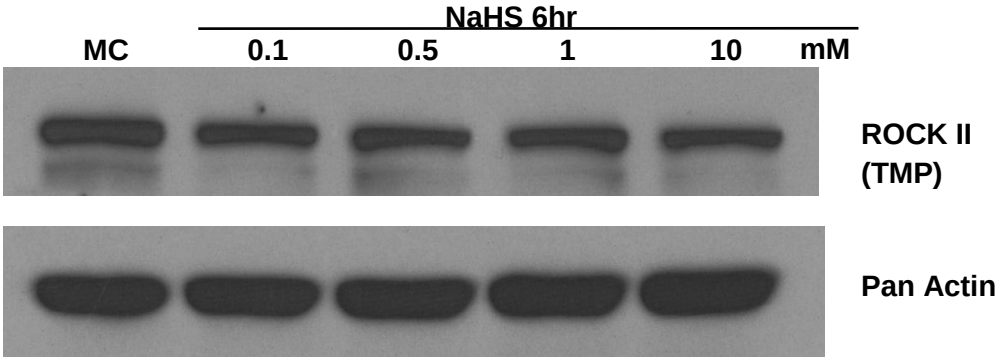


Figure 4.14b RhoA protein expression in rat corpus cavernosum (cytosolic fraction) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=5-7). * $P < 0.005$ (decrease).

Corpus cavernosum treated with NaHS had a lower protein expression of ROCK II. The membrane fraction of ROCK II was reduced to approximately half of its control level in NaHS-treated group (Figure 4.15a) whereas the cytosolic fraction was reduced to less than 40% of its control value (Figure 4.15b). The inhibitory effect of NaHS on the protein expression of membrane ROCK II was apparent at a lower dose (0.1 mM) than on cytosolic ROCK II. While NaHS generally did not have any effect on ROCK II mRNA (Figure 4.13), it had significant effect on ROCK II protein expression (both on the membrane as well as in cytosol). Generally, the trend observed with ROCK II was consistent with that observed with RhoA; H_2S only seemed to act at the level of protein but not mRNA for these two components of the RhoA/Rho-Kinase pathway.



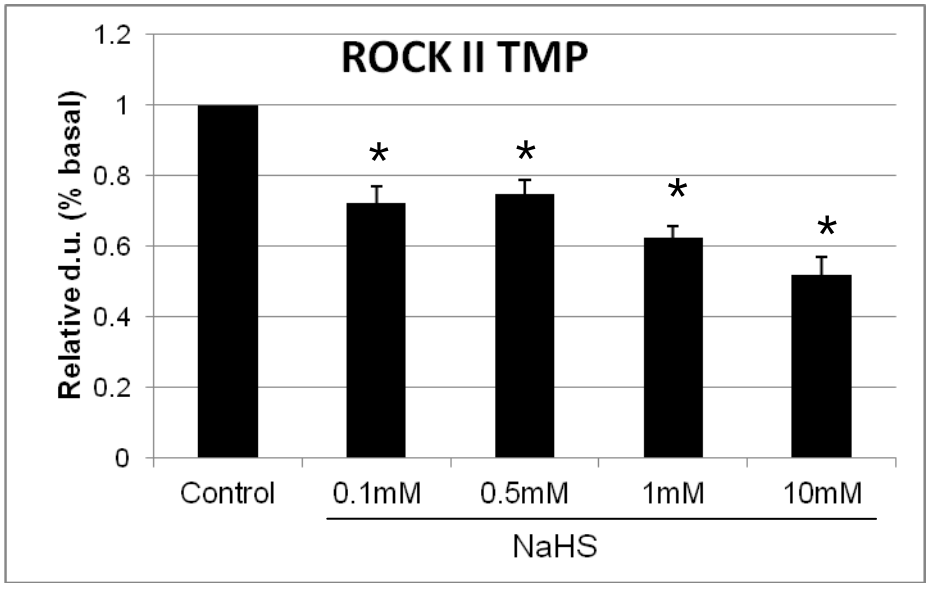
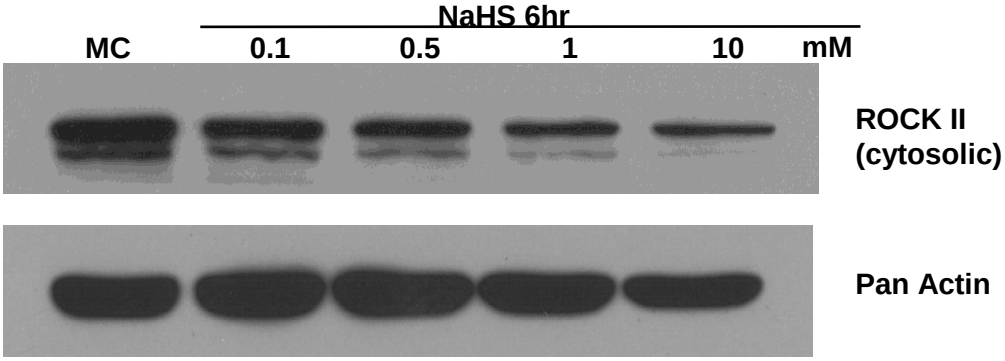


Figure 4.15a ROCK II protein expression in rat corpus cavernosum (TMP) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control pan actin. Data shown are mean ± SEM (n=3-5). *P < 0.001 (decrease).



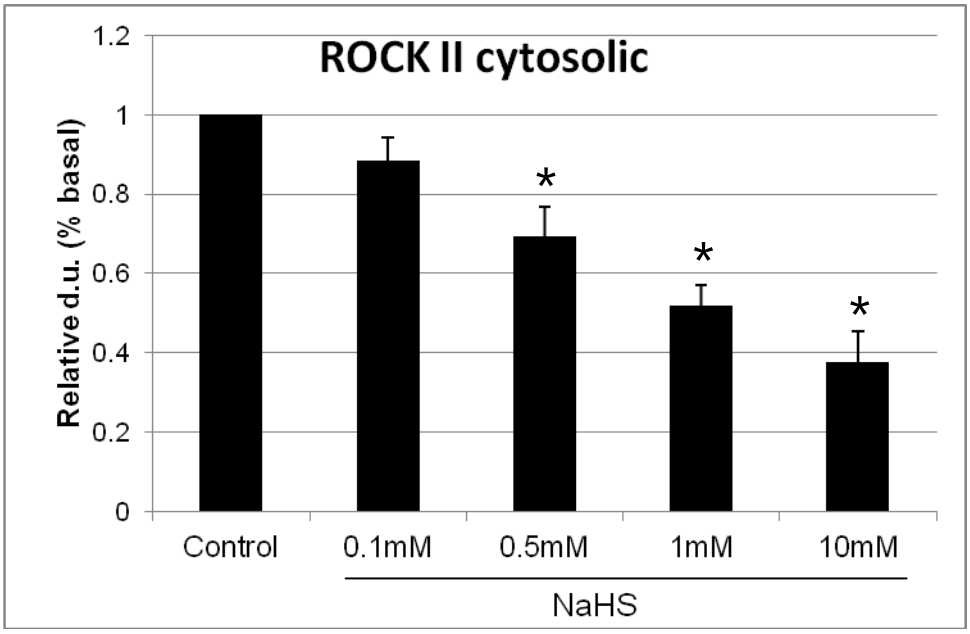


Figure 4.15b ROCK II protein expression in rat corpus cavernosum (cytosolic fraction) in control and NaHS treated group. Results are expressed in relative densitometric unit (d.u) as a percentage of medium control level, after normalizing to loading control Pan actin. Data shown are mean ± SEM (n=4-8). *P < 0.005 (decrease).

4.9 Effects of testosterone

To investigate the involvement of testosterone on the effect(s)/ mechanism of action(s) of H_2S, a comparative study was done between normal rats, castrated rats as well as normal/castrated rats with testosterone supplement. As expected, all the rats castrated were found to have low/negligible amount of plasma total testosterone of < 0.2 ng/ml compared to normal rats, which had an average total testosterone of 2.75 ± 0.5 ng/ml (Figure 4.16). Rats treated with NaHS had a similar amount of total testosterone (3.04 ± 0.2 ng/ml) in plasma, as control/untreated normal rats (2.75 ± 0.5 ng/ml) suggesting that NaHS did not affect testosterone production. Furthermore, all of the castrated rats receiving NaHS treatment had very low plasma testosterone level (< 0.34 ng/ml) which was similar to the testosterone level found in non-treated castrated rats (< 0.2 ng/ml). This also suggests that NaHS had no effect on testosterone production. In both normal/untreated and castrated rats, testosterone

supplements increased the plasma testosterone level to >10 ng/ml (well beyond the normal level), suggesting that the supplement successfully restored the plasma testosterone level in castrated rats.

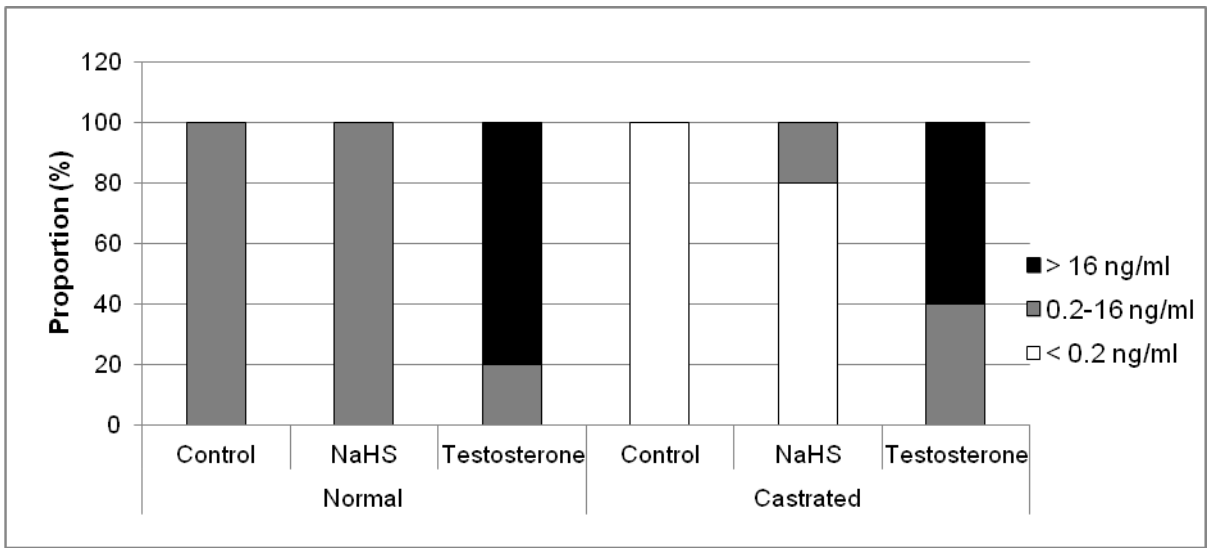


Figure 4.16 Effects of castration and treatment on plasma total testosterone level. Each bar represents the proportion of samples with different concentration of testosterone in the respective treatment group (n=5).

The magnitude of erectile response to electrical stimulations was generally much lower than normal in castrated rats, as indicated by the ICP/MAP ratio of 0.16 ± 0.01 compared to 0.36 ± 0.02 in normal rats, which constituted a 55% reduction. NaHS pre-treatment increased the erectile response in both normal and castrated rats (Figure 4.17). This accentuation appeared to be higher in normal (43%) compared to castrated rats (30%). This was in contrast to testosterone supplements, which only improved erectile response in castrated but not normal rats despite these two groups having a similar testosterone level (> 10 ng/ml); castrated rats treated with testosterone had a higher ICP/MAP ratio of 0.28 ± 0.01 ($P < 0.05$) compared to untreated/castrated control with ICP/MAP ratio of only 0.16 ± 0.01 whereas normal rats treated with testosterone had a comparable ICP/MAP ratio (0.38 ± 0.02) to untreated/normal control rats (with ICP/MAP ratio of 0.36 ± 0.02; $P > 0.05$).

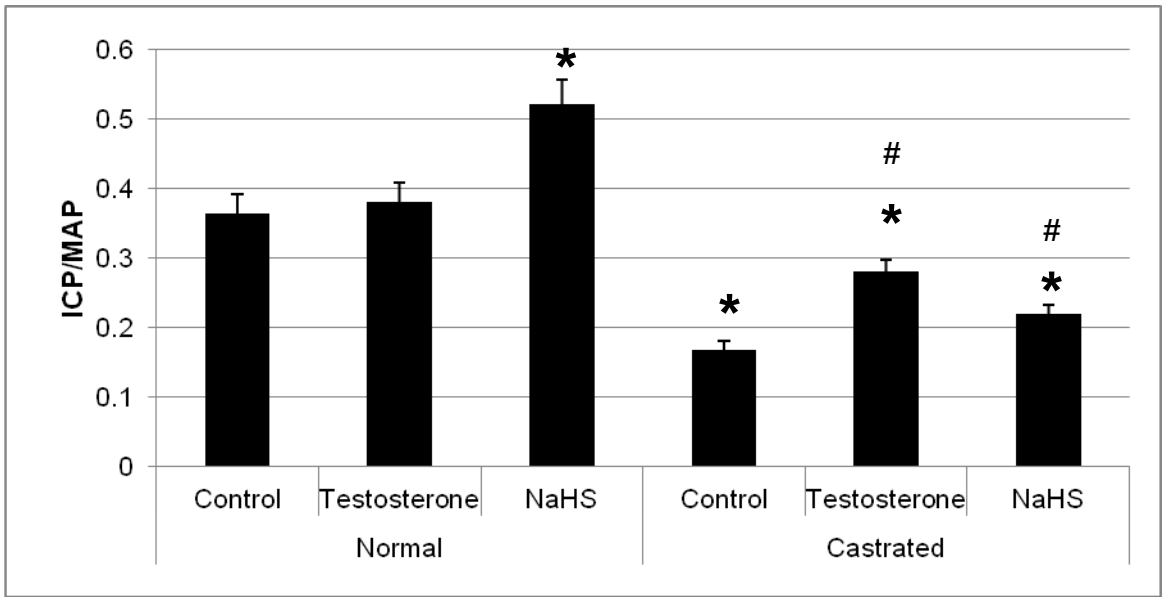


Figure 4.17 Effects of NaHS and testosterone treatment on the magnitude of erectile response (ICP/MAP) in normal and castrated rats. Each bar represents the mean ± SEM of measurements made in 5-7 animals. * $P < 0.05$ (increase/decrease) compared to normal control. # $P < 0.05$ (increase) compared to castrated control.

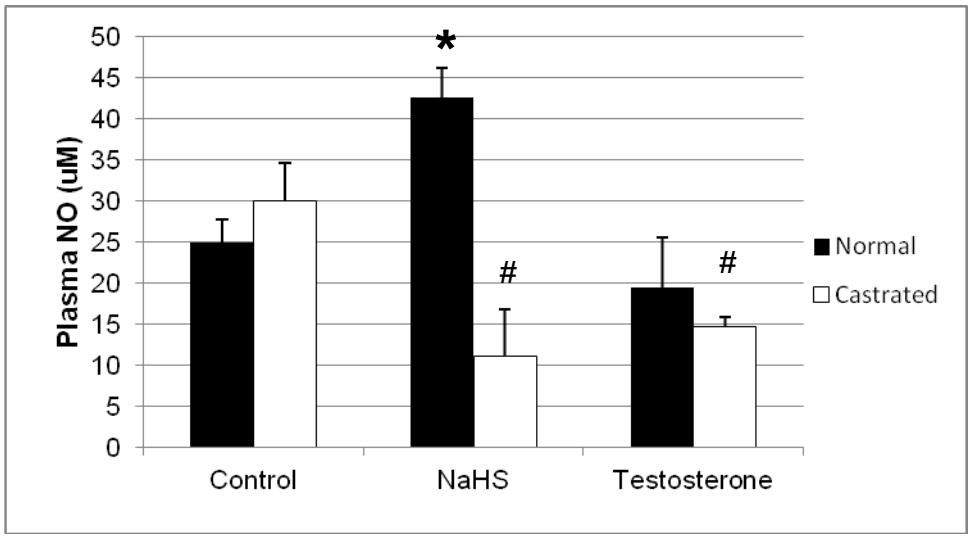


Figure 4.18 Effects of NaHS and testosterone treatment on plasma NO concentration. Each bar represents the mean ± SEM of measurements made in 5-7 animals. * $P < 0.05$ (increase) compared to normal control. # $P < 0.05$ (decrease) compared to castrated control.

The effect of NaHS on plasma NO level was different in normal and castrated rats. Treatment with NaHS increased the plasma NO concentration (from 24.9 ± 2.7 µM in control to 42.5 ±

6.0 μM in NaHS-treated group, $P < 0.05$) in normal rats but decreased plasma NO (from 30.0 ± 4.5 in castrated control to 11.0 ± 1.1 μM in castrated NaHS-treated group, $P < 0.05$) in castrated rats (Figure 4.18).

4.10 Summary of results

1. NaHS and sildenafil treatment *in vivo* increased the magnitude of erectile response (ICP/MAP) to electrical stimulation while L-NAME had the opposite effect.
2. NaHS and sildenafil treatment *in vivo* increased the NO level in plasma and CC.
3. NaHS treatment *in vivo* increased the H_2S level in plasma and CC.
4. NaHS increased the cGMP and cAMP level in CC *in vitro*.
5. NaHS increased the eNOS mRNA expression in CC *in vitro*.
6. NaHS increased the sGCα1 and sGCβ1 mRNA expression in CC *in vitro*.
7. NaHS treatment *in vitro* increased the expression of membrane-bound sGCα1 but decreased that of cytosolic sGCα1 protein in CC.
8. NaHS treatment *in vitro* increased the expression of both membrane-bound and cytosolic sGCβ1 protein in CC.
9. NaHS treatment *in vitro* had no effect on RhoA and ROCK II mRNA expression in CC.
10. NaHS treatment *in vitro* decreased the expression of both membrane-bound and cytosolic RhoA and ROCK II proteins in CC.
11. NaHS treatment *in vivo* increased ICP response in both normal/untreated and castrated rats.
12. NaHS treatment *in vivo* increased plasma NO level in normal/untreated rats but not in castrated rats.

5. DISCUSSION

5.1 Effects of H_2S on erectile response

Earlier studies showed that H_2S exhibited pro-erectile effects in primates and rabbits (Srilatha *et al.,* 2006; Srilatha *et al.,* 2007). Here, it was shown that this pro-erectile effect was also present in the rat model. Treatment with H_2S heightened the erectile response to electrical stimulation in rats (Figure 4.1), even after taking into account H_2S' effects on blood pressure. This is consistent with the finding by Villa Bianca and co-workers where direct, bolus injection of NaHS into the corpus cavernosum caused an increase in the intracavernosal pressure (d'Emmanuele di Villa Bianca *et al.,* 2009). This study however, measured erectile response at the end of a long term (10 weeks) NaHS treatment. Therefore, the result is likely to be a measure of erectile function when the drug is at a 'steady state' concentration, following a chronic/regular dosing and after taking into account any elimination from the body by metabolic processes. This suggests that H_2S can significantly improve erectile function and that when administered regularly in the long term, its metabolism still results in sufficient quantity of 'active agents' in the body to promote erectile function. To verify this, the H_2S concentration and biosynthesis in the plasma and corpus cavernosum of these animals were measured. This will be discussed later in this section.

Both NaHS and sildenafil magnified the ICP rise to electrical stimulation significantly; however, while sildenafil at the dose used in this study had little effect on blood pressure, NaHS caused considerable hypotension (Figure 4.1). This is not surprising considering that H_2S is a vasorelaxant agent (Webb *et al.,* 2008; Bhatia, 2005) and that the deletion of CSE can cause hypertension in mice (Yang *et al.,* 2008). At therapeutic level, this can be a potential drawback similar to PDE-5 inhibitors, if H_2S were to be used for treatment of ED, particularly in patients on nitrates. Identifying analog(s) of H_2S that may have a split of activity (pro-erection vs hypotension) would be an answer to this concern.

It was demonstrated here that L-NAME significantly reduced the magnitude of erectile response, thereby confirming the active involvement of the NO/cGMP pathway in mediating erectile response. It was also shown that L-NAME caused hypertension which is consistent with data in the literature (Küng *et al.,* 1995; Zicha *et al.,* 2003) and expected since NO can regulate systemic vascular resistance and therefore affect blood pressure (Stamler *et al.,* 1994). Treatment with PAG caused a slight reduction in ICP but its ICP/MAP ratio was not significantly different from that of control i.e. it had little/no effect on erectile response at the dosage used in this study.

5.2 Relationship between H_2S, NO and erectile function

This study showed that the increase in NO concentration after treatment with NaHS was correlated with a significant improvement in erectile function (Section 4.2, Figure 4.2). Considering that NO is the main gasotransmitter involved in erectile physiology (Burnett *et al.*, 1992), it is likely that H_2S improved erectile response by influencing the NO/cGMP pathway. NaHS-treated animals (shown to have an elevated level of H_2S than control) exhibited considerably higher systemic and local (CC) level of NO compared to control animals, suggesting that H_2S can facilitate the NO pathway, possibly by increasing the NO production and this increase may be partly responsible for the pro-erectile effect of H_2S. This ability of H_2S to elevate the NO level is suggestive of a cross talk between H_2S and NO. Indeed, the presence of such a cross talk has been demonstrated in the vascular system earlier (Whiteman and Moore, 2009). The results from this study demonstrate the existence of this cross talk in the corpus cavernosum.

It is evident that regular treatment with NaHS for 10 weeks resulted in an elevated level of H_2S in the plasma as well as increased H_2S-synthesizing capacity in the corpus cavernosum (Section 4.3). Since the measurement of H_2S production in the CC was done by quantifying

the rate of release of H_2S when the tissue (containing CSE/CBS) (Srilatha *et al*., 2008) was supplied with the enzyme's substrate (L-cysteine) as well as co-factors, this experiment measured the tissue's 'H_2S-synthesizing capacity' and it was found that given sufficient and equal amount of substrate/co-factors, NaHS-treated animals possessed greater H_2S-synthesizing capability than non-treated (control) animals. This suggests a positive feedback mechanism, where H_2S becomes a stimulator of its own production resulting ultimately in a significant elevation of the H_2S level. It is likely therefore that the observed rise in plasma H_2S was due partly to an accentuated CSE/CBS activity, stimulated by the exogenous H_2S. Similarly, a recent study also demonstrates that onion extract (a rich source of sulphide) can increase endogenous H_2S production in rat aorta (Li *et al.,* 2011). Furthermore, studies on cardiovascular disease models have demonstrated that exogenous H_2S increased CSE expression and activity, resulting in higher H_2S level and production (Chunyu *et al.,* 2003; Yan *et al.,* 2004). The question of whether H_2S treatment in this study increased endogenous H_2S production through activity or expression of the CSE or CBS remains to be answered; further investigation is needed to verify this positive feedback mechanism.

In the present study, the plasma H_2S concentration ranged from 22-42 µM in all the treatment groups (Figure 4.3), which was well within the range reported by other groups (Section 1.3.1.2). It has been shown that even in the presence of > 30 µM of HS^-, no disturbance in oxidative phosphorylation is observed since H_2S gets rapidly oxidised in the mitochondria (Wang, 2002; Bartholomew *et al.,* 1980; Nicholls and Kim, 1981) i.e. at this concentration, H_2S does not disturb cellular respiration to cause toxicity. Therefore, at the range of H_2S concentration observed in this study (22-42 µM) H_2S is unlikely to be toxic. It is possible that the positive feedback mechanism of H_2S may be an evolutionary adaptation which serves as one of the ways in which H_2S can counter its rapid oxidation by the mitochondria to accumulate sufficient quantity to exert a functional effect.

Since the improvement in erectile function with NaHS treatment was correlated with both an increase in NO and H_2S level/production (Section 4.1-4.3), it was difficult to pinpoint the relative contribution of each component to the improvement in erectile response (whether it was due to direct effect of H_2S alone or due to the indirect effect of H_2S on the NO/cGMP pathway or a combination of both). Further study to investigate this relative contribution may utilise a combination of NaHS and L-NAME treatment to determine if inhibition of NO production in the presence of H_2S still results in a significant improvement in erectile response.

Unlike NaHS, the improvement in erectile response that was observed in the sildenafil group was correlated only with increased NO level in plasma and corpus cavernosum; no elevation in H_2S plasma level or H_2S synthesizing capability was observed in this group. There have been reports of sildenafil increasing the expression of eNOS and iNOS mRNA and proteins (Das *et al.,* 2005; Salloum *et al.,* 2003; García-Cardoso *et al.,* 2010) in cardiac myocytes and mononuclear cells. Udenafil, an agent similar to sildenafil, has also been reported to increase the expression of eNOS and nNOS gene and protein in rat model of chemical diabetogenesis (Ahn *et al.*, 2009). The increase in NO level that is observed in this study may be the result of such activation of NO-producing enzymes as shown by other groups. Taken together, it appears that the pro-erectile effect of sildenafil in a clinical setting is probably due to a combination of an elevated NOS activity and its PDE inhibitory activity downstream. What is interesting is the fact that while NaHS treatment (which increased H_2S level and production) changed the NO level, sildenafil treatment (which increased NO level in plasma and CC) did not change the H_2S level/production in this study; however in the vascular system, NO increased H_2S production through stimulation of CSE (Zhao *et al.,* 2001). Thus, the relationship between H_2S and NO appears to be complex and there is currently no specific consensus on the exact nature of their interaction.

As seen from the results (Figure 4.1), L-NAME pre-treatment significantly reduced the magnitude of ICP response to electrical stimulation, an objective parameter of erectile function. This reduction was correlated with a decrease in the plasma NO (but not the NO level in CC) and an increase in plasma H_2S (but not H_2S-biosynthesizing capacity in CC) (Figure 4.2A & 4.3A). The reduction in plasma NO together with the rise in blood pressure that was observed in this group indicated that the L-NAME successfully inhibited NOS and blocked NO production. What these results suggest was that the reduction in NO level was probably partly responsible for the impairment in ICP response that was observed (Figure 4.1). Secondly, high H_2S level (~42 µM compared to 25 µM in control) in this L-NAME-treated group did not seem to be sufficient to reverse/block this impairment. Overall, it appears that while H_2S plays a role in erectile function, the NO/cGMP pathway probably is the major contributor for the cavernous-nerve mediated erectile response.

How L-NAME caused an increase in plasma H_2S level was not evaluated in this study but a similar phenomenon has been documented; L-NAME treatment upregulated CSE protein expression which was accompanied by a rise in H_2S production (Chuah, 2009; Rong-na *et al.*, 2011). At this juncture, the possibility that other enzymes (CBS or MPST) besides CSE may contribute to this increase in H_2S level, still cannot be excluded. In present study, L-NAME failed to lower the NO level in CC even though it successfully inhibited NO production at the systemic level. Since the drug was administered *in vivo*, this lack of local effect by L-NAME is likely to be a pharmacological effect of the drug i.e. failure of the delivery of the drug to the target organ (Lin and Lu, 1997). In this case, an *in vitro* study may be more useful in determining the effect of L-NAME at the tissue level.

In summary, high level of NO (observed in sildenafil group) was associated with normal H_2S level and production while low level of NO (observed in L-NAME group) was associated with high H_2S level. High level of H_2S (observed in NaHS group) on the other hand was associated with increased NO level. These observations are intriguing because they seem to

suggest that H_2S may act as a backup system when the NO/cGMP pathway is compromised for example under pathological conditions such as endothelial dysfunction. One may hypothesise that the shortage of NO would trigger the production of H_2S (possibly by upregulating CSE expression) and when H_2S reached a certain level (quite possibly through positive feedback of its own formation), it would trigger the production of NO. This dual regulation system would have two advantages: firstly, H_2S on its own has very similar properties to NO (i.e. both are vasorelaxants) and therefore by fulfilling the same function as NO, H_2S can serve as a temporary system when there is shortage of NO until the NO level can be restored; secondly H_2S functions to restore the NO level. In the context of erectile physiology, this second function is essential because high level of H_2S in presence of low NO (observed in L-NAME group) appeared insufficient to restore erectile function back to normal. However, considering the importance of the NO/cGMP pathway, the redundancy provided by H_2S may confer survival advantage from an evolutionary perspective. The idea of H_2S acting as 'back up' for NO has also been broached by Li and others before; the authors proposed the existence of 'endothelium-derived H_2S' which may have a similar function to endothelium-derived NO (Li *et al.,* 2009a; Sanderson, 2009).

Although an acute bolus administration of PAG (50 mg/kg) in rats had significantly lowered the ICP response in an earlier work from this lab (Srilatha *et al.,* 2006), in this study, the chronic administration of PAG (50 mg/kg; twice weekly for 10 weeks) failed to lower the measured response to nerve stimulation (ICP) significantly. In fact, the PAG-treated rats also exhibited normal plasma and tissue H_2S levels in this study. It follows that PAG failed to sufficiently inhibit H_2S production and subsequently (either as a direct or indirect result) failed to impair erectile response. It may be important to note here that PAG is an inhibitor of CSE i.e. it would not inhibit other H_2S-synthesizing enzymes such as CBS or MPST (Figure 1.2). Thus, the failure of PAG to inhibit H_2S production and erectile response may be due to the following: 1) since only one dose (50 mg/kg, ip, twice weekly for 10 weeks) was used it was not possible to exclude the possibility that PAG failed to inhibit CSE simply because its

effective concentration was not reached; Zhu *et al* used PAG at the same dose (50 mg/kg, ip) but with higher frequency (daily, for 7 days) (Zhu *et al.*, 2007), this group's dosing regimen had to be modified because a daily administration of PAG at this dose for 10 weeks (as opposed to the short period of 7 days) resulted in very high mortality rate; 2) any decrease in endogenous H_2S production caused by the inhibition of CSE may be compensated by other H_2S-producing enzymes as mentioned above. Considering that PAG failed to inhibit H_2S production in general, its effect on NO level was also likely to be non-specific or even direct (i.e. not caused by low endogenous H_2S). Although PAG inhibited CSE specifically by binding to the enzyme's pyridoxal phosphate binding site, it may also exert effects on other enzymes. However, a search of the available scientific information to date failed to show any specific effect of PAG on NO synthesis.

5.3 Effects of H_2S on the cGMP and cAMP second messenger system

Cyclic GMP is an important cellular mediator of NO signalling. This role is generally established from three main lines of evidence: 1) excellent correlation between the manipulation of cGMP level by endogenous NO and/or NO donor and the pharmacological effect (relaxation); 2) consistent findings that cGMP analogues and cGMP-specific PDE inhibitors mimic NO-dependent relaxations; and 3) the effects of sGC inhibitors in blocking NO-mediated relaxation (Ignarro, 2000).

The binding of NO to sGC increases the activity of the enzyme which would subsequently increase the cGMP level (Ignarro, 2000). In the cell, cGMP may bind to three different kinds of intracellular receptor proteins namely: 1) cyclic nucleotide-dependent protein kinases e.g. protein kinase A (PKA) and PKG; 2) cyclic nucleotide PDE, primarily at allosteric sites on the enzymes; and 3) ion channels, especially those whose activities are amplified upon cGMP binding (Lincoln and Cornwell, 1993). Alternatively, cGMP may also get hydrolyzed by PDE enzymes (Juilfs *et al.*, 1999). Generally, the effect of cGMP would depend on the expression

of specific receptor proteins. The corpus cavernosum expresses all three of these proteins and therefore the effect of cGMP in this context may have far reaching consequences as discussed below.

To investigate whether the effect of H_2S extends to the downstream mediator of the NO pathway, present study looked at its effect on cGMP at the cellular level *in vitro*. In CC, H_2S was shown to cause a dose-dependent increase in cGMP level (Figure 4.4). In this respect, it confirms one of the earlier findings (Srilatha *et al.,* 2008) where H_2S was shown to elevate cGMP concentration in the rat CC. Bucci and co-workers also demonstrated that H_2S elevated cGMP concentration in rat aortic smooth muscle cells and an over-expression of CSE in increased the intracellular cGMP level in a PAG-sensitive manner (Bucci *et al.*, 2010).

This effect of H_2S on cGMP is significant because elevation of cGMP level has been known to be largely responsible for the relaxation of smooth muscle needed to produce erection (Kirby, 1999; Juilfs *et al* 1999). In fact, an increase in cGMP level is physiologically relevant in the context of erectile function because cGMP can activate PKG whose substrates, in turn are directly involved in the modulation of smooth muscle tone, in particular relaxation. Some of the physiologically important PKG substrates include: 1) MBS of MLCP which can modulate the activity of MLCP (PKG phosphorylates the MBS of MLCP to activate the enzyme) (Surks *et al.,* 1999); 2) HSP-20, a 20kDa heat shock-related protein which can modulate smooth muscle contractility (Beall *et al.,* 1997); and 3) phospholamban (PKG phosphorylates phospholamban to increase the Ca^{2+} uptake into the sarcoplasmic reticulum of smooth muscle cells, thereby lowering the Ca^{2+} level in cytosol and subsequently inducing relaxation) (Vrolix *et al.,* 1988; Raeymaekers *et al.,* 1988; Cornwell *et al.,* 1991; Karczewski *et al.,* 1992). PKG can also phosphorylate telokin to accelerate MLC dephosphorylation, giving rise to 'Ca^{2+} desensitization' (Wu *et al.,* 1998). Another important PKG substrate would be K_{Ca} channel α subunit; activation of K_{Ca} channel by PKG would result in efflux of K^+, giving rise to hyperpolarization and inhibition of voltage dependent Ca^{2+} channels in the

smooth muscle cell membrane, eventually resulting in relaxation (Alioua *et al.*, 1998; Dora *et al.*, 2002). In summary, cGMP through the action of PKG regulates the intracellular Ca^{2+} level using several mechanisms of action. However, there is also evidence that cGMP can act as Ca^{2+} mobilizing messenger in a PKG-independent manner (Murthy and Makhlouf, 1998). Further studies are needed to investigate if the H_2S-induced elevation of cGMP is accompanied by an increase in the activity or expression of PKG. Cyclic GMP can also activate PKA, however the level of cGMP in the cell normally fails to reach a high enough concentration (≥ 100 μM) (Wyatt *et al.*, 2003) to cross-activate PKA (Ignarro, 2000).

Interestingly, the H_2S-induced elevation in cGMP concentration observed in this study occurred within a very short span of 30 minutes, suggesting that it could be partly responsible for the acute effect of H_2S that was observed in other studies where intracavernosal injection of NaHS *in vivo* caused an almost instantaneous increase in ICP (d'Emmanuele di Villa Bianca *et al.*, 2009). The very rapid response that H_2S elicited here also suggests that modulation of cGMP level by H_2S in this case was probably through changes in enzyme activity rather than expression. Since the assay in this study employed the use of PDE inhibitor IBMX, this increase was most probably caused by stimulation of the activity of sGC rather than by inhibition of PDE. There exists a distinct possibility that H_2S may directly act on sGC to sensitise it towards NO by binding to an unknown allosteric site on sGC to modulate its responsiveness to heme ligand and regulate its catalytic rate. The benzylindazol derivative YC-1 is able to induce the sensitivity of sGC to NO through allosteric regulation, proving the existence of such sites on the enzyme which can regulate its activity (Ignarro, 2000). The clinical implication of such direct stimulatory effect on sGC is interesting because it represents a promising new therapy for patients with ED who fail to respond to sildenafil or in cases where endogenous NO production is compromised to such an extent that inhibition of cGMP degradation alone has no beneficial effects. However, the possibility that H_2S elevates cGMP by inhibiting PDE also cannot be ignored. Cell-free assay shows that NaHS can inhibit cGMP-PDE activity to a level that is comparable to inhibition by IBMX (Bucci *et al.*, 2010).

The effect of NaHS in increasing cGMP level is also significantly heightened in COS-7 cells overexpressing PDE5 with the converse being true (overexpression of CSE increases the cGMP level in a PAG-sensitive manner while knockdown of CSE reduces the intracellular cGMP in rat aortic smooth muscle cells) (Bucci *et al.*, 2010). Two possibilities as to how H_2S may inhibit PDE activity exist: 1) PDEs are Zinc (Zn)-containing enzymes and removal of Zn abolishes their activity (Bender and Beavo, 2006); H_2S has been known to bind to Zinc (Zn) (Szabó, 2007) and modulate the activity of Zn-dependent enzymes and 2) H_2S may regulate the activity of PDE through post translational protein modification such as sulfhydration (Mustafa *et al.*, 2009).

To investigate the definitive cause of the net increase in cGMP, whether it was due to an increase in cGMP production or an accumulation due to inhibition of its breakdown, a comparative study of the effect of H_2S on cGMP level in presence and absence of IBMX may be useful. Alternatively, the effect of H_2S on cGMP in presence and absence of sGC inhibitor e.g. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) can also be investigated.

It was not clear whether the effect of H_2S in increasing the cGMP level was as a result of a direct effect of H_2S on sGC/PDE and/or an indirect outcome of H_2S' effect in increasing the level of NO. The *in vivo* segment of this study focused on the effect of long term and 'steady state' concentration of H_2S wherein H_2S was found to increase the NO level at week 10 post-treatment. However, the minimum length of time it may take for H_2S to achieve this effect was not known. Nevertheless the possibility that H_2S increased cGMP level by increasing the level of NO cannot be excluded.

It seems that H_2S also increased cAMP level, although this only occurred with a particular dose (1 mM). An increase in cAMP can effectively contribute to erectile response; as a matter of fact PGE1 has been used as a relatively effective treatment for ED (Virag and Adaikan, 1987; Heaton *et al.*, 2001). However, while higher level of cAMP than cGMP was observed

in CC, the effect of H_2S treatment on cAMP level seemed to be much less pronounced than on cGMP and in this case it was unknown if this H_2S-induced increase in cAMP was sufficient to contribute significantly to erectile response.

The concentration of cyclic nucleotides needed to activate PKG generally depends on the phosphorylation state and isoform of the enzyme; PKG can be activated either by high concentration of cAMP or low concentration of cGMP (Ignarro, 2000). This implies that by increasing the cGMP level by 3 folds, H_2S is likely to have important physiological implications. What is interesting to note is that NO seems to be involved in the regulation of PKG; chronic exposure to NO-donors at high concentration has been shown to suppress PKG mRNA in vascular smooth muscles (Soff *et al.,* 1997). Given that H_2S can induce NO production, it is not too far-fetched to suggest that H_2S may act as both stimulator and regulator of the cGMP/PKG signal. The ability of H_2S to induce cGMP production serves as further evidence of the existence of cross talk between H_2S and NO/cGMP pathway in CC.

5.4 Effect of H_2S on eNOS

Studies investigating the cross talk between H_2S and NO pointed out the involvement of eNOS (Chuah, 2009). In the present study, it was found that H_2S increased the expression of eNOS mRNA by as much as 4 folds in the span of 6 hours (Figure 4.6). There was also some indication that this increase may be time-dependent. Endothelial NOS may contribute partly to the H_2S-induced increase in NO level. Considering that eNOS upregulation is time dependent, it was possible that chronic/long term treatment with H_2S led to sustained increase in eNOS expression which could subsequently contribute to the NO pool. Coincidentally, *in vivo* treatment with H_2S also elevated the NO level in plasma and CC (Figure 4.2).

Study by Kubo and others shows that H_2S can inhibit recombinant eNOS (Kubo *et al.,* 2007a), which may appear contradictory to the finding in this study (which demonstrated that

H_2S increased NO production). Their study however, was done in a cell free system *in vitro* (as opposed to *in vivo*) and therefore did not really reflect normal physiological condition where endogenous factors may interfere with this inhibition e.g. in presence of excess NOS co-factor BH_4, NaHS failed to inhibit eNOS (Kubo *et al.,* 2007b). Secondly, the effective concentration range of NaHS which was shown to inhibit eNOS in this enzyme assay was consistent with dose that caused contractile activity in the tissue bioassay (Kubo *et al.,* 2007a). Thirdly, eNOS inhibition alone would not necessarily result in a reduction of the total pool of NO since both nNOS and iNOS can also contribute to this pool (Bruckdorfer, 2005). In fact, the finding by Kubo *et al* is controversial considering that there have been conflicting reports; for example NaHS was found to increase the expression of phosphorylated (at position Serine 1177) eNOS which was consistent with eNOS activation, in ischemia-reperfusion study (Yusof *et al.,* 2009). An independent group has also verified this result; H_2S was found to stimulate production of NO by activating eNOS by phosphorylating it at Serine 1177 (Predmore, 2009). It seems that H_2S may have some effects on NOS activity and investigating the effect of H_2S on the expression of NOS alone may be incomplete.

5.5 Effects of H_2S on sGC

Two types of GC have been identified: cytosolic sGC and membrane-bound particulate GC (pGC; it belongs to a group of receptor-linked enzymes with one membrane spanning domain) (Ignarro, 2000). However, unlike the NO-sensitive sGCs, pGCs are not stimulated by NO (Fessenden and Schacht, 1997) therefore this study focused on sGC but not pGC.

Soluble GC has been purified by several groups and is found to be comprised of an α (large) and β (small) subunit (Gerzer *et al.,* 1981; Humbert *et al.,* 1990; Stone and Marletta, 1995). Currently, two isoforms of each subunit have been identified, namely α1, α2, and β1, β2 respectively. The α1/β1 isoforms constitute the predominant/most abundant dimers present and active in mammals; they have been sequenced and cloned from rat, mouse, bovine and

human (Koesling *et al.,* 1990; Nakane *et al.,* 1990; Zabel *et al.,* 1998). In mammalian tissue, α1/β1 isoforms are also distributed ubiquitously in the heart, kidney, lung, muscle, spleen and brain (Sharina *et al.,* 2000; Budworth *et al.,* 1999). Dimers comprising of α2 and β1 have also been identified but they appear to exist preferentially in specialised tissues e.g. placenta (Russwurm *et al.,* 1998) and central nervous system (Russwurm *et al.,* 2001) and comparison of the heterodimers α1/β1 and α2/β1 does not reveal any differences between the two in terms of NO sensitivity, kinetic properties, heme content, and responsiveness towards modulators (Ignarro, 2000). For all these reasons, the scope of this study was narrowed to sGCα1 and β1.

It was found that H_2S moderately increased the sGCα1 mRNA expression in a dose dependent manner in CC; however, such an upregulation was not observed at the protein level (Section 4.7). Instead, H_2S appeared to be involved in the translocation of sGCα1 protein from the cytosol to the plasma membrane. The NO-sensitive sGC was initially thought to be entirely cytosolic and hence it was termed 'soluble' GC. Recent studies have challenged this designation of sGC as a purely cytosolic enzyme. Approximately 20% of the sGC in the rat heart for example is found in the membrane fraction (Zabel *et al.,* 2002). Other tissues such as skeletal muscle, colon, brain cortex, cerebellum and adrenal gland have also been found to contain membrane-associated sGC (Zabel *et al.,* 2002). Indeed, this study also demonstrated that such membrane-bound sGC existed in the CC. At moderate to high doses, H_2S increased the expression of membrane-bound sGC α1 protein but decreased the expression of cytosolic sGCα1, suggesting that rather than modulating the overall protein expression of sGCα1, H_2S was involved in the cellular translocation of sGCα1 from cytosol to the plasma membrane. This finding has a significant implication because studies have found that translocation of sGC to the plasma membrane modulates the enzyme's activation properties and sensitises it to NO (the dose-response curve to NO donor is shifted to the left in membrane-associated sGC compared to cytosolic sGC) (Pyriochou and Papapetropoulos, 2005); this higher sensitivity is thought to be related to the higher stability of the membrane-bound sGC and NO complex and regulated by intracellular events such as elevation in intracellular free Ca^{2+} concentration

(Zabel *et al.*, 2002). In the context of endothelial cells, it is hypothesised that the translocation of sGC to the plasma membrane would bring the enzyme closer to the 'sphere of influence' of NO, given that NO has high solubility in lipid, a very short $T_{1/2}$ in aqueous environment and that NO is generated predominantly in plasma membrane in endothelial cells. This H_2S-induced translocation of sGC to the plasma membrane is an intriguing finding given that H_2S was also able to induce NO production (Section 4.2). It appeared that H_2S was greatly capable of enhancing NO signalling through a multipronged approach viz, by boosting the NO level and by sensitizing the sGC to NO at the same time.

Time point study was not done in this case due to several major challenges. It was discovered that the medium used to dissolve NaHS (DMEM) had some effects on sGCα1 protein expression (this was done by comparing the protein expression in untreated CC with that in CC treated with the medium; result not shown). This effect was also observed with other proteins e.g. sGCβ1 (please refer to Figure 4.10). This means that for each time point used, a separate vehicle/medium control was needed i.e. more tissues were required. Considering the limited number of animals and amount of corpus cavernosum tissue available in this study, this was not practical. Moreover, antibiotics were not added to the medium as they can affect protein expression by binding to ribosomes (Pommerville, 2010). Absence of antibiotics increased the risk of bacterial contamination, making studies with long incubation period not ideal.

It was found that H_2S also increased the expression of sGCβ1 mRNA in CC (it is still unclear if this increase was due to an increase in transcription or an increase in the stability of the mRNA). More importantly, this upregulation was reflected at the protein level; H_2S dose-dependently increased the expression of sGCβ1 in both cytosol and plasma membrane. This protein upregulation also appeared to be sustained for at least up to 12 hours. The residues needed for substrate recognition and catalysis are distributed on the two chains of the heterodimeric α1/β1 sGC enzyme; therefore, even though each subunit possesses a catalytic

domain, the presence of both is essential for enzymatic activity. Nevertheless, most of sGC's regulatory features are determined by the β1 subunit. Experiments using truncated subunits reveal that the N-termini of both α1 and β1 subunits are required for proper heme binding (Foerster *et al.,* 1996; Ignarro, 2000). However, these 2 subunits appear to contribute unequally, with β1 being the primary heme binding subunit (Zhao and Marletta, 1997); mutation on cysteines 78 and 214 of the β1 subunit yields sGC with lower heme-binding affinity, whereas mutation on equivalent residues on the α1 subunit does not alter the enzyme's sensitivity to NO (Friebe *et al.,* 1997). This prosthetic heme group serves as the acceptor site for NO on the sGC (Humbert *et al.,* 1990; Stone and Marletta, 1995) and its presence is required for the stimulatory effect of NO (Craven and DeRubertis, 1978; Ignarro *et al.,* 1982; Ohlstein *et al.,* 1982). Histidine-105 residue of the β1 subunit has been identified as the axial ligand of the heme group (Zhao *et al.,* 1998; Wedel *et al.,* 1995). There is evidence that it is the displacement of this axial ligand by NO that triggers the conformational change which results in the increase in enzyme turnover/activity (Martin *et al.,* 2003; Dierks *et al.,* 1997; Ignarro, 2000). Therefore, the increase in sGCβ1 expression that was induced by H_2S potentially represents an increase in sGC's 'heme binding capacity' for NO i.e. H_2S is likely to contribute to the regulation of sGC by altering the enzyme's sensitivity towards NO.

What is interesting to note here is that H_2S had distinct effects on the different subunits of sGC; it induced translocation of the α1 subunit but increased the expression of the β1 subunit. Considering that the enzymatic activity of sGC requires the presence of both subunits, it is still unclear whether this increase in the expression of the β1 subunit alone is sufficient to contribute to higher production of cGMP by the sGC. Further studies on the localization of both subunits would prove to be useful in determining whether the α1 subunit co-localise with β1, in particular whether the translocation of α1 to the plasma membrane is accompanied by the β1 subunit and also in determining where the 'extra' β1 subunits (obtained as a result of the upregulation) is localised.

Regardless of whether or not H_2S increased the net activity of sGC eventually, it is quite clear that H_2S is involved in the regulation of sGC, in particular in modulating the enzyme's sensitivity towards its ligand NO. Moreover, it was found that NO-activation of sGC was potentiated under reducing conditions e.g. in presence of ascorbate, dithiothreitol, glutathione or cysteine (Ignarro, 2000). With pKa of 7.04, H_2S at the physiological pH of 7.4 can essentially provide a reducing environment in the body which potentially can contribute to the potentiation of sGC's activation by NO. This means that physiologically, as H_2S increased NO production (Figure 4.2), it may also enhance the ability of the NO generated to potentiate the activation of sGC at the same time.

5.6 Effects of H_2S on RhoA/Rho-Kinase pathway

As mentioned in section 1.3.2.5, RhoA is a small monomeric GTPase; it is inactive in its GDP-bound state and activated when it is bound to GTP. These two forms of RhoA exhibit different localization profiles. Agonist induced activation of G-protein-coupled receptor (GPCR) brings forth an exchange of GDP for GTP on RhoA (Webb, 2003). The enzyme guanine nucleotide exchange factors (RhoGEFs) cause dissociation of RhoA from its binding partner, Rho-guanine dissociation inhibitor (RhoGDI). This causes the inactive RhoA in the cytosol to translocate to the membrane (Bokoch *et al.*, 1994; Gong *et al.*, 1997b; Gong *et al.*, 1997a). One of the downstream targets of RhoA is the serine/threonine protein kinase, Rho-Kinase (ROCK) (Leung *et al.*, 1995; Amano *et al.*, 1996). Two isoforms of ROCK have been identified in the mammalian system: ROCK I (also known as ROKβ or p160ROCK) (Ishizaki *et al.*, 1996) and ROCK II (also known as ROKα) (Leung *et al.*, 1996). The RhoA/Rho-Kinase pathway has been implicated in erectile physiology, in particular in the maintenance of the flaccid or contracted state of the penis through 'Ca^{2+}-sensitization mechanism' involving MLCP (Jin and Burnett, 2006).

As previously discussed in section 1.3.2.4, smooth muscle relaxation can be brought about either by a decrease in intracellular Ca^{2+} concentration or through 'Ca^{2+}-desensitization' of the contractile apparatus. This means that a thorough investigation on the mechanism of action of H_2S in CC should include both its effect (facilitatory) on relaxation of the corporal smooth muscle as well as its effect (inhibitory) on the contractile pathway (since both may give rise to pro-erectile effects).

The present study found that generally, H_2S had no effect on the mRNA expression of both RhoA and ROCK II, while ROCK I was hardly detected in CC (Section 4.8.1). However, H_2S reduced the expression of RhoA protein in both cytosol and plasma membrane (Section 4.8.2). This means that not only did H_2S downregulate the general expression of RhoA protein but it also effectively reduced the expression of active RhoA. Moreover, this H_2S-induced decrease in RhoA was correlated with a downregulation of both cytosolic and membrane-bound ROCK II protein, suggesting that H_2S' effect on RhoA successfully resulted in modulation of the downstream mediators in this pathway and was likely to have some functional implication. ROCK II proteins are normally localised in the cytosol under resting state and are translocated to the membrane upon activation by Rho (Leung *et al.,* 1995); this means that H_2S not only downregulated the general expression of ROCK II but it also reduced the expression of the active ROCK II (an effect that is similar to that observed with RhoA). This finding is important because MLCP is an important downstream target protein of ROCK II. The MLCP holoenzyme is comprised of 3 subunits: a small 20 kDa non catalytic subunit (M20), a catalytic subunit of type 1 phosphatase (PP1) and a 100 kDa MBS (which is also known as myosin phosphatase target subunit or MYPT) (Hartshorne *et al.,* 1998). ROCK II has been found to phosphorylate MBS of MLCP at threonine 695/697 (chicken/rat) and threonine 850/855 (Kawano *et al.,* 1999). Phosphorylation of Thr 695 seems to be required and sufficient for inhibition of the catalytic activity of PP1 (Feng *et al.,* 1999), while phosphorylation of Thr 850 dissociates PP1 from myosin which also renders the MLCP inactive (Velasco *et al.,* 2002). This means that H_2S-induced reduction in ROCK II

expression/activity was likely to increase the activity of MLCP, giving rise to smooth muscle relaxation.

The ROCK II can also directly phosphorylate serine 19 residue of the MLC, which is the same residue that gets phosphorylated by MLCK (Amano *et al.,* 1996); phosphorylation of this residue is essential in facilitating the activation of myosin ATPase by actin (Kamisoyama *et al.,* 1994; Bresnick *et al.,* 1995). Therefore, it appears that ROCK II can increase cellular contractility via dual effects on MLC and MLCP and this will effectively modify the sensitivity of smooth muscle contraction to changes in Ca^{2+} concentration. Considering the role that ROCK II plays in smooth muscle contraction, it is likely that the downregulation of ROCK II by H_2S will contribute substantially to smooth muscle relaxation.

In general, H_2S seemed to affect the RhoA/Rho-Kinase pathway at translational but not transcriptional level. In this study, it was also shown that when administered *in vivo*, H_2S improved erectile response i.e. the pro-erectile effect of H_2S appeared to be associated with downregulation of RhoA and ROCK II proteins in the CC. This possibility is supported by the finding that membrane-bound (active) RhoA is higher in aged compared to young rats and is correlated with lower ICP/MAP (Jin *et al.,* 2006). Moreover, inhibition of RhoA activity by over-expressing dominant negative RhoA in CC improves erectile function (Jin *et al.,* 2006; Chitaley *et al.,* 2002).

The reduction in RhoA and ROCK II protein expression observed in this study can be correlated to an increase in eNOS mRNA expression as well as cGMP level in CC. Studies have shown that not only is RhoA/Rho-Kinase pathway involved in erectile biology, but it is also likely to be interlinked with the NO pathway (Pintérová *et al.,* 2011). Rho-Kinase and eNOS are found to co-localise in the endothelium of CC. The penile tissue of streptozotocin (STZ)-induced diabetic rats has higher RhoA and ROCK II protein level but lower eNOS protein and cGMP compared to control. Transfection of these STZ diabetic rats with

adenovirus encoding dominant negative RhoA (which lowers the penile RhoA and ROCK II protein level) restores the eNOS protein and cGMP level to that observed in control (Bivalacqua *et al.,* 2004). The ROCK II mRNA and protein are also found to be upregulated in the CC of eNOS knockout mice (Priviero *et al.,* 2010). Moreover, there is also evidence that the RhoA/Rho-Kinase pathway is implicated in the vasorelaxation induced by NO and vice versa. Both L-NAME and ODQ shift the dose response curve of Rho-Kinase inhibitor Y-27632 to the right while Y-27632 enhances SNP-induced relaxation in coeliac artery smooth muscle (Teixeira *et al.,* 2005). On the other hand, cGMP/cGK pathway can inhibit RhoA and block RhoA-induced Ca^{2+} sensitization in vascular smooth muscle since cGK can phosphorylate RhoA at serine 188 residue and cause the translocation of membrane-bound RhoA to the cytosol, rendering it inactive (Sauzeau *et al.,* 2000). There is also evidence that cGMP can bring about phosphorylation of serine 695 (human isoform) of the MLCP, which effectively increases the cayalytic activity of MLCP by blocking phosphorylation at threonine 696 (the human isoform of rat's Thr 697) (Wooldridge *et al.,* 2004). This means that in this study, H_2S - by elevating the cGMP level - may indirectly cause the translocation RhoA to the cytosol (and therefore its inactivation) through the action of cGK or directly activate MLCP by blocking the inhibition caused by phosphorylation at Thr 697 residue.

The results presented here were consistent with evidence in the literature i.e. lower RhoA expression was associated with higher eNOS and cGMP level in CC. However, the cause and effect relationship between RhoA and NO/cGMP is still unresolved. With the observed effects on both pathways, it is unknown if H_2S first affected (downregulated) RhoA and/or Rho-Kinase which then caused the upregulation of eNOS and cGMP level or if the downregulation of RhoA was a result of H_2S-induced upregulation of eNOS and cGMP level.

Moreover, it also appears that RhoA/Rho-Kinase signalling has an NO-independent component since inhibition of this pathway (while sufficient to cause relaxation of the corporal muscle) is not completely blocked by inhibitors of NOS (L-NAME) and sGC

(methylene blue) (Chitaley *et al.,* 2001). This is not surprising considering that ROCK II can affect MLC and MLCP directly (as has been discussed above), without any apparent requirement for the presence of either NOS or sGC.

In general, the improvement in erectile function brought about by H_2S may be related to: 1) its ability to interfere directly with the RhoA/Rho-Kinase pathway - by downregulating RhoA and ROCK II - which gave rise to inhibition of the contractile activity of the corporal smooth muscle, 2) its ability to strengthen NO signalling (by upregulating eNOS, increasing NO production and cGMP level) which gave rise to corporal smooth muscle relaxation or 3) a combination of both, which involves a 'cross-talk' between RhoA/Rho-Kinase and NO pathway.

5.7 Effects of testosterone

To study the nature of the cross talk between H_2S and NO, the role of testosterone in the relationship between the two pathways was investigated. Castration did not completely abolish the rise in ICP to electrical stimulation but it caused a significant decline in the magnitude of erectile response (Section 4.9). This is consistent with previous report which shows that erectile response is comprised of both androgen-dependent and independent components (Mills *et al.,* 1994). In this study, it was observed that in normal rats, NaHS improved erectile response and this was correlated with an increase in plasma NO, in presence of normal level of total testosterone. In castrated rats, NaHS also improved erectile response in presence of low/negligible amount of total testosterone, but this was not correlated with a high plasma NO.

In other words, H_2S increased the magnitude of erectile response in both normal and castrated rats, suggesting that testosterone is not a requirement for the pro-erectile effects of H_2S. However, H_2S appears to be more effective in improving erectile response in normal

compared to castrated animals. This may be attributed to several factors: 1) testosterone may act specifically to enhance the responsiveness of vascular smooth muscle to neurotransmitters/gasotransmitters as was shown in the study by Mills and co-workers (Mills *et al.*, 1992), 2) testosterone may improve the relaxation of the vascular smooth muscle which regulates blood flow into the cavernous sinuses during erection (Mills *et al.*, 1996), 3) testosterone may directly increase endogenous H_2S production by increasing the enzymatic conversion of L-cysteine to H_2S (Bucci *et al.*, 2009), which together with the exogenous H_2S supplied would elevate the final H_2S concentration in the body.

Interestingly, testosterone supplements improved erectile response only in castrated rats but not normal rats. It appears that testosterone is important for erectile function but that a maximum threshold of testosterone level or 'saturation point' exists where any addition beyond this point does not improve the erectile function anymore i.e. only testosterone in physiological range exerts beneficial effect. In the present study, H_2S did not seem to affect testosterone production, suggesting that while testosterone may confer some pro-erectile effects on its own (as discussed above), the pro-erectile effects of H_2S was not as a result of testosterone effects. In summary, H_2S did not require testosterone for its pro-erectile effects but these effects may be augmented in presence of testosterone.

After castration and/or testosterone treatment, changes in plasma NO were not statistically significant, suggesting that testosterone level is unlikely to be a modulator of the plasma NO level (Figure 4.18). Castration lowered plasma testosterone, which was associated with a significant reduction in erectile response (but not plasma NO) and this impairment in erectile response could be restored partially by treatment with NaHS and/or testosterone supplement. It was interesting to note that while H_2S improved erectile response in both normal and castrated animals, it increased plasma NO only in normal animals. In fact, it was observed that H_2S reduced plasma NO in castrated rats. This suggests that the role of H_2S in erectile function may differ in physiological and pathological conditions and that while H_2S may

improve erectile response in normal animals by increasing NO production, it seems to improve erectile function in castrated animals through other means. Testosterone is clearly implicated in the cross talk between H_2S and NO but the mechanism of action of H_2S appears to be different depending on the testosterone level. In low testosterone environment (such as that induced by castration), H_2S seems to improve erectile response through an NO-independent mechanism.

6. CONCLUSION

The results of this study lead to the proposition that the pro-erectile effect of H_2S is achieved through multiple mechanisms of action (Figure 6.1). Firstly, H_2S seemed to be involved in a cross talk with the NO/cGMP pathway; it was capable of increasing NO production, eNOS mRNA expression as well as cGMP level in the cavernosum. Testosterone is likely to be implicated in this cross talk. More specifically, high testosterone level seemed to favour the cross talk while low testosterone seemed to cause H_2S to 'switch' to an NO-independent mechanism for its pro-erectile effect. Moreover, the local hormonal milieu could also influence the 'magnitude' of the H_2S-induced improvement in erectile function. With respect to NO pathway in erectile physiology, H_2S seemed to play a 'supportive' role, amplifying NO signalling through dual action of increasing NO production and sensitizing the sGC towards NO. Interestingly, it appeared that H_2S could auto-regulate its own production through a positive feedback and potentially, acted as a backup when the NO pathway was compromised.

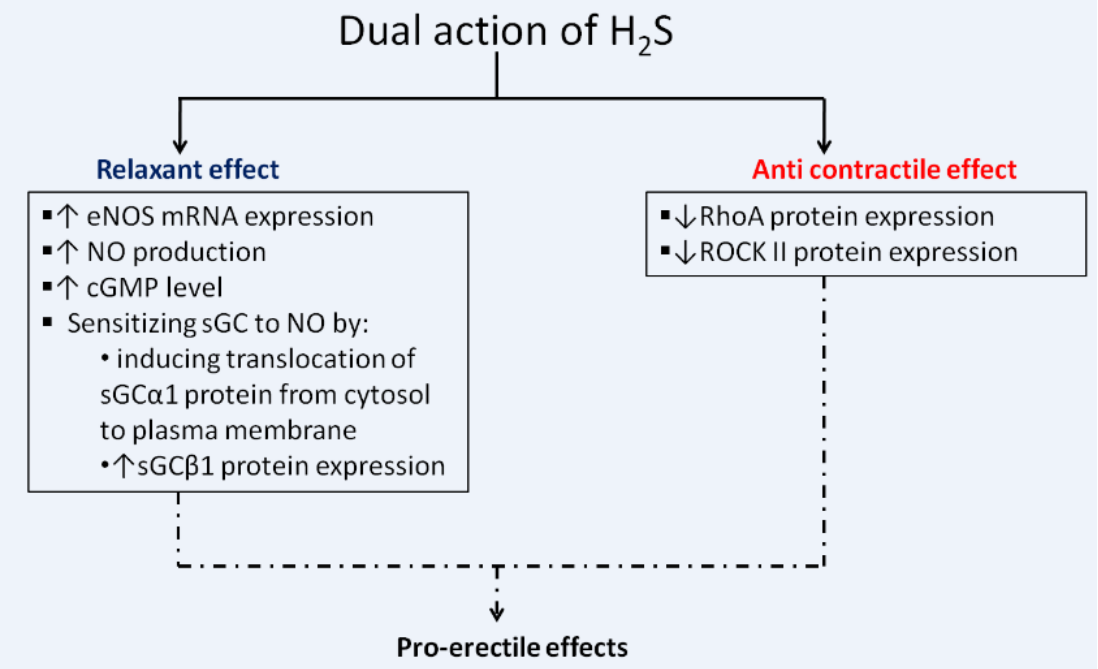


Figure 6.1 Relaxant and anti contractile effects of H_2S

Secondly, H_2S was involved in the downregulation of important components in the contractile pathway viz, RhoA and ROCK II proteins. Looking at the myriad of possible mechanisms of action of H_2S, one may propose that the effect of H_2S (and its mechanism of action) may differ depending on the local environment e.g. the relative expression of K^+_{ATP} channels, PDE isoforms expressed, amount of cAMP/PKA, cGMP/PKG and H_2S in a particular tissue. Future work can look at the relative contributions and importance of the different mechanism of H_2S action in erectile physiology.

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